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It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

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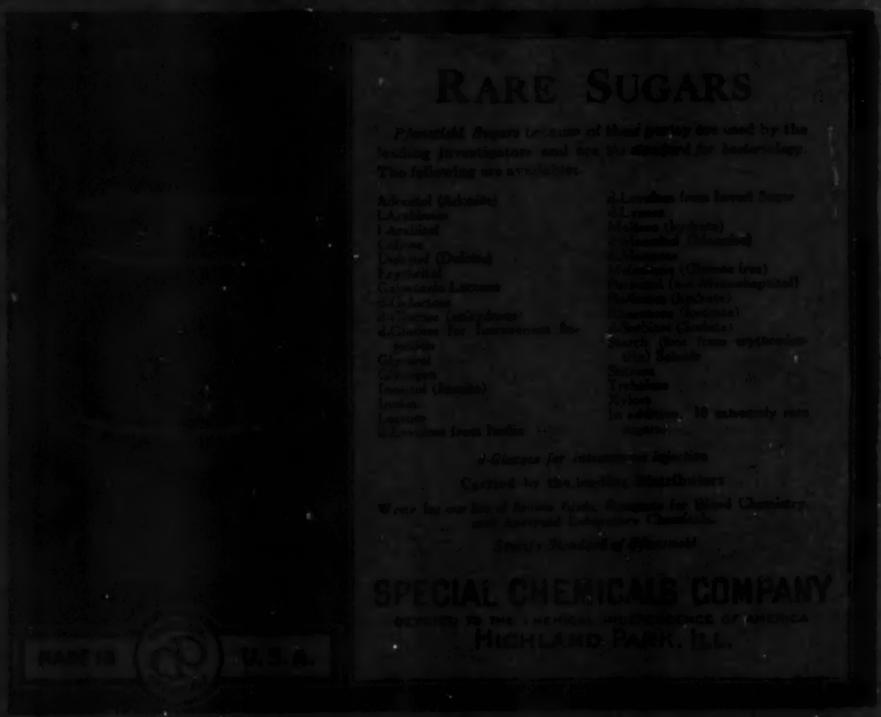

THE DETECTION OF BACILLUS BOTULINUS AND BACILLUS TETANI IN SOIL SAMPLES BY THE CONSTRICTED TUBE METHOD

IVAN C. HALL AND EMELIA C. PETERSON[1]

From the Department of Bacteriology and Experimental Pathology University of California, Berkeley, California

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The researches of Tanner and Dack (1922) and of Meyer (1922) and his collaborators have shown beyond question that *B. botulinus* is widely distributed in the soil and that its presence therein can be proven by testing the toxicity of mixed cultures made in a peptic digest medium.

Our own first attempts to demonstrate toxin forming anaerobes in the soil, using glucose peptone broth in the constricted tube with marble seal, were total failures although pure culture controls of *B. botulinus* and *B. tetani* were always highly toxic. We finally showed that the failures were due to the lack of sufficient buffer in our glucose broth, so that fermentative bacteria present in the soils destroyed the toxin as fast as formed and perhaps to some extent inhibited growth of the anaerobes in question. Owing to the well known high resistance of botulinus toxin to acid alone it became evident that some other factor, incidental to growth, and other than acid, but dependent upon it, was responsible. At any rate, the inclusion of ground meat enabled us to secure filtrates of high toxicity from soil cultures to which we had added pure cultures of *B. botulinus* (Hall and Peterson, 1923) and *B. tetani*.[2] We believe that the value of Meyer's peptic digest medium resides

[1] Aided by a grant from the Board of Research of the University of California and by the provision of a University Fellowship for the junior author during the year 1922–23.

[2] Unpublished data.

largely in its high buffer content and that equally good results may be more easily secured by our method.

While our first lots of meat mash medium were sterilized in the Arnold sterilizer we later found that sterilization in the autoclave at 20 pounds for thirty minutes was equally effective providing the glucose (15 per cent aqueous) was separately and similarly sterilized and then added in quantity sufficient to make a final concentration of 1 per cent. Sterilization in the autoclave of meat mash medium containing glucose always produced a dark colored product of inferior quality for bacterial growth.

Having found in these experiments certain conditions satisfactory for the detection of tetanus and botulinus toxins in soil cultures to which these organisms had been added we next proceeded to retest with meat mash medium in the constricted tube some of the same soil samples which in the earlier tests had all failed to reveal toxic filtrates, and were gratified to learn that a large majority contained either *B. botulinus*, or *B. tetani*, or both.

TECHNIC

The technic followed was similar to that already described in our first paper (1922) except that the soil inocula were not weighed but were measured upon a sterile spatula holding about one gram. Following incubation at 37°C. for two or three days, the cultures were filtered through small ($\frac{1}{2}$ by $2\frac{1}{2}$ inch) Berkefeld or Mandler filters. The meat particles of the medium did not interfere with filtration. Sterility and toxicity tests were conducted as described in our first paper.

RESULTS

Table 1 shows certain details regarding the sources and character of the soils and the tests performed with them.

Of 20 samples, 14 (70 per cent) gave toxic filtrates. *B. botulinus*, type A toxin was found alone in 7 specimens, in conjunction with type B in 2 specimens, and with tetanus once, a total of 10 (50 per cent). Type B was found alone once, and with type A, twice, a total of 3 (15 per cent). Tetanus toxin was found alone 3

times and with type A once, a total of 4 (20 per cent). Six (30 per cent) of the soils gave filtrates non toxic in doses of 1 cc.; larger doses were not tested.

It is easy to identify a monotypic filtrate containing tetanus toxin by the peculiar symptoms and specific protection with tetanus antitoxin, or one containing one or the other of the several types of botulinus toxin in a similar manner.

In typing botulinus filtrates we used three guinea pigs, all of which were injected with a certainly lethal dose of toxin. One served as an unprotected control and each of the others, received either type A or type B antitoxin. The one that survived indicated the type of toxin tested. It is unnecessary to present our protocols upon these tests for the method is well known (see Hall, 1922).

In the case of samples 8 and 15, it was found that all three test pigs died of botulism. This finding suggested the following possibilities: first, that the antitoxin used was too weak, second, that both types A and B were present, or third, that a type other than A or B might be present. The second was shown to be the correct explanation by tests using the same amounts of filtrate and of both A and B type antitoxic serums as in the first tests; pigs so protected survived while the controls injected with single antitoxins died. These tests also eliminated the first and last hypotheses.

In the case of sample 9 peculiar symptoms in the pig suggested that both tetanus and botulinus toxins were present. Tetanospastic paralysis of the legs indicated tetanus while lack of tone in the abdominal muscles suggested botulism. We had previously performed some fantastic experiments with balanced mixtures of tetanus and botulism toxins in the wild speculation that one might neutralize the other, since the visible symptoms of these two most powerful bacterial poisons are so unlike. Of course neutralization does not occur but the experimental symptoms of both diseases are considerably altered when they are made to occur together, and the alteration is just as above described. There was no difficulty at all in typing this filtrate for botulism toxin, type A, by means of three pigs all protected

TABLE 1

Bacterial toxins in soil cultures in California

SOIL	LOCALITY	DESCRIPTION	INCUBATION PERIODS	TOXIC FILTRATES	GUINEA PIG WEIGHTS	DOSES AND SYMPTOMS	ANTITOXIN TESTS
			days		*grams*		
1	Stockton	Peat	2	−	360	1 cc.—Non-toxic	
2	Stockton	Peat	2	−	200	1 cc.—Non-toxic	
3	Santa Barbara	Sandy loam	2	+	260	1 cc.—Botulism—dead 36 hours.	*B. botulinus*—type A
4	Santa Barbara	Sandy loam	3	+	270	1 cc. (1:100) Chronic botulism—killed eighth day.	*B. botulinus*—type A
5	Walnut Creek	Clay	3	+	400	1 cc. (1:100) Botulism—recovered.	*B. botulinus*—type A
6	Walnut Creek	Clay	3	+	250	1 cc. (1:1000) Botulism, dead 3 days.	*B. botulinus*—type A
7	Walnut Creek	Fine sand	3	+	330	1 cc.—Dead 18 hours	*B. botulinus*—type A
8	Walnut Creek	Sandy loam	3	+	340	1 cc.—Dead 24 hours	*B. botulinus*—types A and B
9	Earlimart, Tulare County	Sandy loam	3	+	470	1 cc.—Symptoms mixed, dead 48 hours	*B. botulinus*—type A and *B. tetani*
10	Earlimart Tulare County	Gravelly adobe	3	+	390	1 cc.—Tetanus—Dead 48 hours	*B. tetani*
11	Orland Glenn County	Gravelly loam	3	−	520	1 cc.—Non-toxic	
12	Orland Glenn County	Gravelly loam	3	−	540	1 cc.—Non-toxic	

*13	Gardena	Sandy soil	3	+	640	1 cc.—Botulism—Dead 4 days	*B. botulinus*—type A
14	Gardena	Sandy soil	3	−	610	1 cc.—Non-toxic	
15	Gardena	Sandy soil	3	+	400	1 cc. (1:10)—Botulism—dead 2 days	*B. botulinus*—types A and B
16	Palo Alto	Clay	3	+	260	1 cc.—Botulism—dead 2 days	*B. botulinus*—type A
17	Palo Alto	Clay	3	+	600	1 cc. (1:10)—Botulism 48 hours—recovered	*B. botulinus*—type B
18	Huntington Park	Fine sand	2	−	530	1 cc.—Non-toxic	
19	Huntington Park	Fine sand	2	+	570	1 cc.—Slight tetanus fifth day—recovered	*B. tetani*
20	Bodega	Fine sand—some clay	2	+	470	1 cc.—Slight tetanus ninth day—recovered	*B. tetani*

* This soil gave a non-toxic filtrate in the first trial.

with tetanus antitoxin, and for tetanus by protecting another series with botulism A antitoxin also.

DISCUSSION

It would be impossible statistically to compare so small a series of examinations with the results based upon the much larger number of samples reported by Tanner and Dack (1922) and by Meyer and his collaborators (1922). If we disregard for the moment, however, the considerable discrepancy in number of samples examined it will be seen that although all of our soil samples were derived from areas at least potentially arable the proportion of toxic cultures was considerably higher than reported by Meyer in any of his series and was only exceeded by the small series of tests made by Coleman (1922) who found that 8 (89 per cent) out of 9 soils from a restricted area in California were toxic. We note also that while *B. botulinus* type B has been demonstrated mainly in eastern soils (Tanner and Dack, 1922) our own results, like those of Meyer *et al*, show the predominance of type A in the West.

Dobovsky and Meyer (1922) found that while 45 (57.6 per cent) out of 78 virgin soils from California gave toxic cultures, only 59 (26.9 per cent) of 226 cultivated soils did so. They were unable to identify the type in 40.6 per cent of the toxic cultures derived from cultivated soils but stated that among those typed, A predominated over B, 38:1 in the virgin group and 4.66:1 in the cultivated. Seventeen samples from pasture land also yielded only 7 toxic cultures of which 3 were type A, 1 type B, and 4 could not be typed.

The results for the United States at large (Meyer and Dubovsky, 1922) were similar to those for California alone: 375 (24.3 per cent) out of 1538 specimens of soils and soil products were toxic. It is particularly unfortunate that in this large series the actual identity of the toxic agents could not be determined in almost a third of the tests showing evidence of toxin.

Similar comments might be made upon the results of these authors with soils from Alaska and Canada, certain European

Countries, and Hawaii and China (1922). It seems to us that the large proportion of toxin forming samples in all of these soils in which the nature of the toxin was unidentified vitiates the value of any conclusions regarding the actual ratio of *B. botulinus* types in them and leaves any speculation as to the origin of one type from another by mutation, environmental influences, or other evolutionary process, entirely unsupported. We say this without prejudice to our belief that evolution operates for bacteria according to the same laws that apply to higher forms and in particular that mutation is a demonstrable phenomenon for bacteria. But we cannot accept Meyer's data as proof of mutation.

We also appreciate the ingenuity with which Coleman's results, obtained under Dr. Meyer's supervision and by similar methods, were harmonized on the grounds of propinquity to the mountains. But how can ours be harmonized with his hypothesis? For our samples were derived from widely separated areas in California.

Surprising and illuminating as were the data collected by Dr. Meyer and his associates in showing the widespread distribution of *B. botulinus* we believe that his figures are too low, especially considering that his cultures were usually inoculated with relatively large amounts of soil, (in some cases as high as 50 grams) that a prolonged period of incubation (ten days or more) was used, and that the tests were usually not made with bacteria free filtrates but rather with supernatant fluids which were perhaps generally not sterile.

Neither should we be too dogmatic in asserting that *B. botulinus* is not an intestinal form. It is quite possible that the better nutrient conditions of the bowel prevent sporulation and that the methods hitherto used to detect *B. botulinus* in porcine (Burke, 1919) and other feces have failed for technical reasons. Graham and Barger (1921) and Tanner and Dack (1922) have already reported *B. botulinus* in human stools; the former did not state their cultural methods, but the latter certainly cultivated only spores. Further work in this direction with carefully controlled methods is very desirable, not only to determine the

distribution of *B. botulinus* but of other obligate anaerobes, sporulating as well as non-sporulating.

It is curious that in the work reviewed *B. tetani* has not been encountered more often. Dubovsky and Meyer (1922) have pointed out that "no attempt has been made to study the factors necessary to demonstrate this bacillus in mass enrichment cultures." They reported only 19 instances out of a total of 2379 samples of soils and vegetables from the United States, Europe and China, and of 624 California specimens in which "special attention was paid to the occurrence of tetanus bacilli in mountain soil." But we easily demonstrated *B. tetani* in 4 out of 20 samples. Surely we are not yet ready to agree that "the soil of the western states is relatively free from this anaerobe."

SUMMARY

1. Glucose meat mash medium in the constricted tube provides a highly satisfactory method of detecting *B. botulinus* and *B. tetani* in soil through the formation of their toxins.

2. Of 20 samples of arable soil 70 per cent gave toxic filtrates, 50 per cent contained *B. botulinus* type A, 15 per cent contained *B. botulinus* type B, 20 per cent contained *B. tetani* and 30 per cent gave no evidence of toxin forming anaerobes.

3. The wide distribution of *B. botulinus* in California soils and the predominance of type A is confirmed but we are unable to agree that the evidence proves the derivation of one type from the other.

4. *B. tetani* is by no means rare in arable soils of California.

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THE DISCOLORATION OF BRAIN MEDIUM BY ANAEROBIC BACTERIA

IVAN C. HALL AND EMELIA PETERSON[1]

From the University of California, Berkeley, California

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Late in 1922 we noticed that our stock cultures of *B. welchii*, *B. novyi*, *B. tetani* and *B. putrificus* were blackening our brain medium, although the first two had never done so previously and the latter two only tardily. All were known however to blacken brain medium early and distinctly in the presence of added iron (Hall, 1922).

Our first inclination was to suspect contamination by actively putrefactive anaerobes of which there are several that blacken brain medium even though no iron is added. But it was impossible to detect any such contamination and we therefore began to suspect iron as an impurity in some ingredient of the medium. This indeed proved true of the pepton and since blackening of brain and other protein mediums is of considerable importance in the differentiation and identification of the sporulating anaerobes and since the mechanism of this phenomenon has aroused some speculative interest, our results are presented herewith.

HISTORICAL

There is no little obscurity regarding the earliest use of animal tissues in the cultivation of obligate anaerobes. Gunning (1878) and Nencki (1879) studied the chemistry of anaerobic putrefaction, but, if they observed blackening of their meat cultures, failed to mention it. Gunning, however, observed and recorded the pale red color now known to be produced by old cultures

[1] Supported in part by a grant from the Board of Research of the University of California.

of *B. welchii* under certain conditions. Smith (1890) used small pieces of liver in the fermentation tube but made no reference to blackening, due perhaps to the use of 2 per cent glucose in the medium which would have protected the protein from attack.

The first recorded observation of the blackening of putrefied meat may be attributed tentatively to Tissier and Martelly (1902). It was again noted by Rettger (1906) who also remarked that "hydrogen sulfide is, no doubt, one of the first substances . . . . split off from the proteid molecule during putrefaction," and that "even the heating of proteids in a neutral or slightly alkaline solution at 80°C. will bring about the elimination of hydrogen sulfide." We agree with this in so far as it applies to alkaline solutions or suspensions.

No immediate use of the phenomenon of blackening for differential purposes appears to have been made although Tarozzi (1905), Wrzosek (1905–1907), and Harass (1906) used various animal tissues for anaerobic cultures under what they called "aerobic" conditions, which must now be recognized as really "anaerobic," owing to the slowness with which oxygen is reabsorbed by particulate suspensions freed from it by boiling, and in the case of fresh unheated tissues, to their reducing action.

Von Hibler (1908) was apparently the first to use the discoloration of animal tissues by anaerobes for differentiation and recognized the general comparability of muscle, liver and brain mediums.

Cooked meat mediums have had perhaps a wider use than brain as appears from a review of papers by Robertson (1916), Henry (1917), Weinberg and Seguin (1917), Adamson (1919), British Medical Research Committee (1917–1919), Holman (1919), and Kahn (1922), and one notes the uniformity of formula, that of Robertson, followed by all of these observers, in which a suspension of ground meat particles is neutralized and sterilized without addition of other ingredients. But while we have tried both Rettger's (1906) egg meat mixture and Robertson's cooked meat medium we prefer brain medium because of its whiteness.

Von Hibler's formula called for ground brain substance to be boiled for one-half to one hour in $\frac{1}{3}$ to $\frac{1}{4}$ volume distilled water

or 0.6 per cent sodium chloride solution, filled into tubes in depths of 5 to 10 cm. and sterilized three or four times in the steamer. No other ingredients were added.

This formula was followed by Meyer (1915) in the United States and by Adamson (1919) in England, only that Adamson used more water. It was modified by one of us (Hall, 1920) through the addition of 0.1 per cent glucose and of 2 per cent pepton.

The added glucose stimulates rapid growth and gas formation and favors the development of some anaerobes that grow poorly if at all except in the presence of fermentable carbohydrates; an excess is to be avoided however as generally inhibiting sporulation and preventing discoloration of the medium by certain organisms through protein sparing action. Excess glucose also leads to excessive acid production and early death of some cultures.

Added pepton improves the medium by increasing its free amino acid and buffer content which may be assumed to favor earlier development of the cultures, more intensive color changes, and prolonged viability. Pepton also gives to brain medium a perfectly clear supernatant fluid, which is an advantage in the detection of contamination prior to inoculation, or of growth afterwards, while brain medium made without pepton is generally slightly turbid or opalescent and permits the detection of contamination or of delicate growth less easily. We now realize however that care must be exercised in selecting the pepton.

In order to elucidate our viewpoint it is necessary to discuss the mechanism of the discoloration of brain and meat mediums. Von Hibler correctly attributed the blackening to the formation of iron sulfide by sulfuretted hydrogen set free from the protein molecule. But he was surely wrong in assuming that the iron is derived from traces of hemoglobin present in the brain tissue, as one of us (Hall, 1921–1922) has already suggested, and as we shall prove herein. Henry (1917) suggested that it might be "a tyrosin derivative, such as that which results from the interaction of tyrosin with tyrosinase; or it may be a black humin substance resulting from the condensation of an amino-body with a sugar." Yet Henry inclined toward the iron sulfide

theory though he had no absolute proof. Hall (1921) has shown that *B. tyrosinogenes* produces both tyrosin and the black discoloration in meat and brain mediums but only tyrosin in a salmon medium; when iron is added to the salmon medium however the blackening occurs as usual. Further evidence of the distant relation between blackening and tyrosin formation is that all actively putrefactive anaerobes blacken meat and brain mediums but only four (*B. bifermentans*, *B. centrosporogenes*, *B. histolyticus*, and *B. tyrosinogenes*) are known to produce tyrosin.

The fact that an excess of fermentable carbohydrate prevents the discoloration of brain and meat mediums certainly eliminates the second hypothesis of Henry and all of our evidence goes to show that the blackening of meat and brain medium is due to the precipitation of iron sulfide by sulfuretted hydrogen acting upon iron derived from sources in the mediums other than hemoglobin.

EXPERIMENTAL

Test organisms were selected for the variety of their proteolytic effects as described in 1922. They were carefully re-examined for impurities but none was found.

The basic medium was made according to our published formula with the omission of pepton. It corresponded therefore to that of Von Hibler with the addition of 0.1 per cent glucose. To this basic medium various test substances were added as shown in the protocols.

All cultures were incubated at 37°C. Growth occurred in each tube as shown by turbidity, and, except for *B. histolyticus*, by gas production. Aerobic contamination was excluded by the usual check tests on agar slants.

Experiment 1. The test organisms were inoculated into three parallel lots of medium as indicated in table 1 and incubated for four days at 37°C. when the readings were made. This experiment was terminated too soon to show blackening of the basic medium by any of these cultures of which the first three are actively putrefactive but there was a perfect correspondence

between the action of iron and of Difco pepton added to the basic medium.

Some tests were made of 1 and 0.5 per cent Difco pepton differing quantitatively in time of appearance and amount of blackening but qualitatively like the above.

Experiment 2. We next tested several brands of peptones, each in a concentration of 2 per cent. The cultures were incubated at 37°C, for forty-five days. Growth was proven in

TABLE 1

Showing the effect of Difco pepton and of iron added to the basic medium (0.1 per cent glucose brain)

	BASIC MEDIUM	BASIC MEDIUM PLUS IRON WIRE	BASIC MEDIUM PLUS 2 PER CENT DIFCO PEPTON
B. sporogenes 10	−	+++*	+++
B. histolyticus 141	−	++	++
B. tyrosinogenes 106	−	+++	+++
B. welchii 2	−	+	+
B. novyi 139	−	+	+
B. tetani 131-2	−	++	++
B. butyricus 24	−	−	−
Control—not inoculated	−	−	−

* The sign + indicates blackening; the sign − indicates failure to blacken.

each culture both by turbidity and microscopic examination, and freedom from aerobic contamination by subculture on plain agar aerobic plates after twenty-four hours incubation. Color changes were determined daily for the first week and thereafter weekly by comparing with the uninoculated control in each set. Table 2 is a résumé of these readings.

We note that only *B. sporogenes*, *B. histolyticus* and *B. tyrosinogenes* blackened the basic medium, or the basic medium plus Parke Davis (two lots), Armour's, or Fairchild's pepton; *B. welchii*, *B. novyi*, *B. tetani*, and *B. butryicus* blackened none of these within forty-five days. The results in these mediums were qualitatively identical, but quantitatively, as indicated by earlier appearance and greater intensity of color, more striking in the presence of pepton. It is suggested that pepton may

accelerate blackening either by furnishing a more accessible source of hydrogen sulfide or by stimulating more vigorous growth, or possibly both. These statements hold also for the basic medium plus Witte's pepton except for the delayed blackening by *B. tetani*.

The delayed blackening by *B. histolyticus* and especially *B. tetani* as contrasted with *B. sporogenes* and *B. tyrosinogenes* was evident in all of the mediums except those containing either Difco pepton or iron wire.

TABLE 2

*Showing the effect of various peptones added to the basic medium (0.1 per cent glucose brain)**

	BASIC MEDIUM	BASIC MEDIUM PLUS DIFCO PEPTON (LOT 10,512)	BASIC MEDIUM PLUS WITTE'S PEPTON (NO LOT NUMBER)	BASIC MEDIUM PLUS PARKE-DAVIS PEPTON (PINE) (LOT 2,480,271)	BASIC MEDIUM PLUS PARKE-DAVIS PEPTON (COARSE) (LOT 2,542,505)	BASIC MEDIUM PLUS ARMOUR'S PEPTON (LOT 105,214)	BASIC MEDIUM PLUS FAIRCHILD'S PEPTON (LOT 220,130)	BASIC MEDIUM PLUS IRON WIRE
B. sporogenes 10	+7	+2	+4	+3	+4	+4	+3	+3
B. histolyticus 141	+15	+2	+15	+15	+15	+22	+22	+2
B. tyrosinogenes 106	+7	+2	+4	+2	+4	+7	+3	+3
B. welchii 2	−45	+7	−45	−45	−45	−45	−45	+2†
B. novyi 139	−45	−45	−45	−45	−45	−45	−45	+3†
B. tetani 1	−45	+1	+30	−45	−45	−45	−45	+15†
B. butyricus 24	−45	−45	−45	−45	−45	−45	−45	−45†
Control—uninoculated	−45	−45	−45	−45	−45	−45	−45	+45

* Blackening is indicated by the sign +, failure to blacken by the sign −, the day of incubation upon which the first blackening was observed, or in those cases where none was observed, the last day of observation, is indicated by the numeral.

† Red oxide of iron.

With Difco pepton these four organisms blackened the medium much more quickly and more intensively. In addition the culture of *B. welchii* was black in seven days, but *B. novyi*, which was one of the species that called our attention to the problem failed to blacken at all and behaved just as did *B. butyricus* in this lot of medium.

With iron brain all but *B. butyricus* became black.

No blackening occurred in any of the uninoculated controls within thirty days; the iron brain medium control had blackened

distinctly however in forty-five days but direct examination of stained and unstained smears, and subcultures failed to show any contamination. The reaction had become quite alkaline. In a subsequent experiment with iron brain we found that if a small amount of sodium hydroxide is added the medium becomes quite black within thirty days and especially so if the tube be sealed, without any evidence of bacterial decomposition; similar tubes left neutral or slightly acidified failed to blacken at all. Even if we admit that the alkali in the uninoculated iron brain tube in

TABLE 3

*Showing the effect of iron added to basic medium in the presence of various peptones.**

	BASIC MEDIUM		BASIC MEDIUM PLUS DIFCO PEPTON (LOT 10,512)		BASIC MEDIUM PLUS WITTE PEPTON		BASIC MEDIUM PLUS PARKE-DAVIS PEPTON (LOT 2,489,271)		BASIC MEDIUM PLUS PARKE-DAVIS PEPTON (LOT 2,542,505)		BASIC MEDIUM PLUS ARMOUR PEPTONE (LOT 105,214)		BASIC MEDIUM PLUS FAIRCHILD PEPTON (LOT 220,130)	
	Iron	No iron	Iron	No iron	Iron	No iron	Iron	No iron	Iron	No iron	Iron	No iron	Iron	No iron
B. welchii 2	+7	−40	+2	+2	+5	−40	+1	−40	+5	−40	+2	−40	+2	−40
B. novyi 139	+14	−40	+2	−40	+5	−40	+5	−40	+5	−40	+2	−40	+2	−40
B. tetani 131	+14	+28	+2	+2	+5	+33	+5	−40	+1	+33	+2	−40	+2	−40
B. butyricus	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40
B. chauvoei 6	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40
Vibrion septique 4	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40	−40
B. tertius 148	−40	−40	−40	−40							−40	−40	−40	−40
Control—uninoculated	−40	−40	−40	−40	−40	−40	+40	−40	+26	−40	−40	−40	−40	−40

* Symbols are same as used in table 2.

experiment 2 was responsible for the blackening we are still unable to explain the development of the alkali. But the hypothesis that the blackening is due to an alkaline hydrolysis of the tissue protein seems a valid one.

The observations growing out of experiment 2 show that the reactions described for the test organisms in Hall's differential key depend upon the kind of pepton used in the brain medium, and that the Difco pepton gives reactions qualitatively resembling those given by iron in the medium, except in the case of *B. novyi*

and we have observed such reactions even with this organism in uncontrolled cultures left for a longer time.

Experiment 3. We next tested the effect of certain of the less actively putrefactive anaerobes upon mediums containing the various peptones, with and without added metallic iron. The details of procedure and observation were similar to those in experiment 2. The results are shown in table 3.

One observes that *B. butyricus*, *B. chauvoei*, *Vibrion septique*, and *B. tertius* failed to blacken any of the mediums. *B. welchii* and *B. novyi* failed to blacken any of those mediums without added iron except that *B. welchii* blackened that containing Difco pepton. *B. tetani* on the other hand blackened quite tardily the basic medium without added iron as well as several of the pepton mediums; it failed also to blacken certain of the pepton mediums without added iron. But with added iron these three organisms, *B. welchii*, *B. novyi*, and *B. tetani*, all blackened the mediums containing pepton quite promptly; the basic medium without pepton but with iron was also blackened but distinctly more slowly and less intensely. These results are in line with all our experience which indicates the improved growth of anaerobes in brain mediums containing pepton and may be explained by the greater accessibility of the nutrients in the pepton as compared with undigested tissue.

Curiously, after two or three weeks incubation *B. butyricus*, *B. chauvoei*, *Vibrion septique*, and *B. tertius* sometimes gave peculiar gray discolorations in pepton or plain iron brain media differing from true blackening in having a peculiar bluish tinge and often a peculiar gray brown color. Simultaneous occurrence of red iron oxide was frequent; whether the oxide or gray discoloration appeared first is not known since after the first few days observations were made only at intervals of a week. The two Parke Davis iron controls were also considerably darkened on the twenty-sixth and fortieth days (table 3), the oxide being present and the reaction alkaline. No contamination could be detected, after a rigid examination as in a similar instance (table 1) already described.

It seems possible that after complete utilization of carbohydrates in old brain cultures of *B. butyricus*, *B. chauvoei*, *Vibrion septique*, and *B. tertius*, 0.1 per cent glucose being present in less than an excess, a very mild proteolytic action may occur or the acids resulting from fermentation may themselves be destroyed neutralizing the otherwise acid medium and even rendering it slightly alkaline. Some hydrolysis of protein may take place with liberation of hydrogen sulfide followed by precipitation of iron sulfiide in the presence of an iron nail.

One must exercise judgment in determining blackening of brain medium with iron. Readings should be made before the appearance of the oxide which may still be found to be associated with the bluish gray discoloration. Time is a factor, and organisms blackening iron brain medium will have produced that change before oxidation occurs.

The detection of iron in pepton and brain tissue

Experiment 4. Following out the suggestion that Difco pepton probably differs from the others in iron content, six peptones were subjected to chemical test for iron. Two 5 cc. portions of 10 per cent solutions of each pepton were rendered acid to litmus with 1 cc. $\frac{N}{10}$ HCl. One cubic centimeter of 2 per cent solutions of potassium ferrocyanide and potassium ferricyanide were added, one to each series. Difco pepton gave an immediate deep blue color with potassium ferrocyanide indicating ferric iron, the same result being obtained in a repeated test by adding only 1 drop of reagent.

Potassium ferricyanide gave a green color, but whether this was due to ferrous or to ferric iron was hard to determine owing to the deep yellow color of the reagent itself.

None of the remaining brands gave any immediate color changes. The slight green tints acquired by some after standing were disregarded as the reagents decompose on exposure to air. Negative results were also obtained by testing a tube of basic medium in this way. But after hydrolyzing with NaOH and acidifying, a light blue color appeared in the depths of the

medium after adding potassium ferricyanide, indicating a trace of ferrous iron.

At this juncture Dr. C. L. A. Schmidt of the Biochemistry Department of this university suggested the ashing method as being more delicate for detecting iron in these substances. Bennett's procedure for demonstrating masked iron in blood was followed with but slight modifications.

Experiment 5. Three to four grams of pepton were ashed with approximately an equal volume of potassium nitrate. After acidifying and dissolving the ash, a fraction was poured into a test tube, ether added and a crystal of ammonium thiocyanide. In this test when iron is present, the ether becomes colored with iron sulphacyanide varying from a pink to a deep wine color. This is decanted into another test tube, a small amount of water being added with a crystal of potassium ferrocyanide. After shaking, a deep blue color appears in the water when iron is present in the ferric form.

Difco pepton gave an immediate deep blue color confirming the previous test, but the other brands also contained traces of iron. The colors varied from a very light green with Parke Davis (coarse), Fairchild, and Armour peptones to darker greens with Parke Davis (fine) and Witte brands, the trace of blue color in these instances being hidden by the yellow color of the reagent.

That different lots of the same brand of pepton vary in their chemical make-up is indicated by our own observations upon two lots of the Parke Davis product. Thompson (1920), Myer (1920) and Tilley (1921–1923) have also emphasized the great variability of peptones in relation to the production of hydrogen sulfide. We emphasize the varying iron content of different lots and brands of pepton and suggest the use for brain medium of pepton as free as possible from iron. Bennett's test should be applied to each lot used.

It is true, of course, that our basic brain medium without pepton or added iron is blackened by the highly putrefactive anaerobes and we have already referred to Von Hibler's belief that this is due to iron derived from the hemoglobin in the brain tissue. We were able to show only a trace of iron by Ben-

nett's test in the ash from a tube of basic brain medium which had been carefully guarded against contact with iron utensils during its preparation. A more definite but still delicate test was obtained by ashing a whole sheep's brain that had been carefully washed in distilled water.

The effect of blood upon the blackening of brain medium

If Von Hibler's view were correct, the addition of blood to brain medium should intensify the blackening effect just as the addition of metallic iron does. This is not the case, however, and this raises the whole question as to what form of iron is available for this reaction.

TABLE 4

*The effect of blood upon the blackening of brain medium**

	BASIC MEDIUM	BASIC MEDIUM PLUS IRON	BASIC MEDIUM PLUS UNHEATED BLOOD	BASIC MEDIUM PLUS HEATED BLOOD
B. sporogenes 10	+13	++2	−22	+13
B. centrosporogenes 76	+13	++2	−22	+13
B. histolyticus	+13	+2	−22	−22
B. tyrosinogenes 106	+13	++2	−22	+12
B. botulinus 8A	+13	++2	−22	+13
Control—uninoculated	−22	−22	−22	−22

* The symbols are the same as in previous tables.

Experiment 6. Table 4 shows the results of an experiment with certain actively putrefactive species in our basic brain medium to which in one series metallic iron was added, in another unheated sterile rabbit blood, and in still another heated sterile rabbit blood. Each set was controlled by an uninoculated tube. Five-tenths cubic centimeter of fresh defibrinated rabbit's blood was added to each tube of the unheated series after boiling and cooling while the same amount was added to the second series before boiling. After twenty-four hours incubation, they were checked for aerobic sterility and observed daily for blackening. This experiment shows again the prompt blackening of brain medium with iron and the delayed blackening of the brain medium without added iron. The medium with unheated blood failed to

blacken at all during the period of observation while those with heated blood blackened approximately the same as the basic medium, or if anything, less intensely.

Iron brain was blackened by all five cultures in forty-eight hours, and very intensely on the sixth day. The basic medium was moderately blackened by all on the thirteenth day. None could be detected with the unheated blood due to the red color which later became brownish green making detection of blackening even more difficult. There was an indication of blackening in all but *B. histolyticus* in the presence of heated blood which did not exceed plain brain in intensity on the twentieth day, but was, if anything, less. These results prove conclusively that the iron in hemoglobin does not intensify the blackening of brain medium and indicate that in so far as iron is responsible it must be derived from compounds of simpler nature, as already pointed out.

DISCUSSION AND CONCLUSIONS

The addition of pepton to brain medium improves its clarity, favors the rapid development of bacteria, intensifies color changes, and prolongs the viability of culture; but cognizance should be taken of the presence of iron in certain lots of pepton in view of the effect of iron upon the blackening of brain medium by some organisms. Difco pepton was found to have an effect upon the blackening of brain medium by *B. welchii*, *B. novyi*, and *B. tetani* similar to that of metallic iron, differing in this respect from all other brands tested. Difco pepton was shown by chemical tests also, to contain a considerable quantity of iron, whereas other peptones contain only a trace of iron.

Brain medium made without pepton also contains iron which is responsible for the blackening of such a medium by certain obligate anaerobes. This iron is derived from the brain tissue and is apparently so firmly bound that only the most actively proteolytic bacteria are able to set it free. There is no evidence that it comes from hemoglobin. The addition of blood to brain medium does not intensify the blackening by any of the test organisms and we may assume that the iron in hemoglobin

is too firmly fixed for even the most actively putrefactive anaerobes to set it free.

The use of blackening in brain medium as a criterion in the differentiation and identification of the sporulating anaerobes should involve a knowledge of the form in which iron occurs in the medium.

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FURTHER OBSERVATIONS ON LIQUEFACTION OF GELATIN BY BACTERIA

MAX LEVINE AND F. W. SHAW

Department of Bacteriology, Iowa State College, Ames, Iowa

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In observing the liquefaction of gelatin by microörganisms bacteriologists have been hampered by lack of adequate quantitative means for measuring this property. The methods commonly employed are unreliable and inconvenient even from a qualitative standpoint.

In a recent paper Levine and Carpenter employed the change in viscosity and formol titration for detecting the gelatinolytic properties of bacteria. They observed that the viscosity (at 25°C.) of autoclaved gelatin when stored at 22°C. changed very little for a few days and then increased markedly. Gelatin inoculated with non-liquefying organisms acted in a similar manner. Proteolytic bacteria, on the other hand, rapidly decreased the viscosity of the medium. The proteolytic types fall into two groups with reference to their formol titration figures; in one group the formol titration increased slowly and did not reach a very high figure, in the other, the rise was very rapid and considerable.

The effect of the increase in viscosity of uninoculated gelatin on storage (or gelatin inoculated with non-proteolytic organisms) is to exaggerate the difference between the proteolytic and non-proteolytic organisms, so that as a qualitative test the change in viscosity at 25°C. may prove to be very convenient and delicate. For comparative purposes, however, it would be far more desirable if the gelatin medium could be manipulated so that its viscosity would remain constant when not proteolized.

It was the purpose of the following experiments to ascertain whether autoclaved gelatin, after storage at various temperatures for prolonged periods, could be treated so as to yield products of constant viscosity.

A. REJUVENATION OF GELATIN

The gelatin solution employed was prepared in the following manner:

To a flask containing 1000 cc. of distilled water at 40°C. was added 20 grams of gelatin and the material set in an incubator at 41°C. for four to five hours. This period appeared adequate for complete solution. The reaction was then adjusted to a point neutral to phenolphthalein with N/1 sodium hydroxide. Ten cubic centimeter portions were placed in tubes and sterilized in the autoclave at 15 pounds for fifteen minutes. When the tubes were again cool enough to handle they were sealed in the flame, so as to eliminate changes due to evaporation.

TABLE 1

Viscosity of 2 per cent gelatin (Difco) at 30°C. after storage.

STORAGE AND TREATMENT	STORED IN ICE BOX AT 10–12°C. FOR SIX DAYS, THEN WARMED AT 30°C. FOR HALF HOUR	STORED IN ICE BOX AT 10–12°C. FOR FOUR DAYS, THEN HEATED AT 41°C. FOR FOUR HOURS. SET AT ROOM TEMPERATURE (26–28°C.) FOR 2 DAYS THEN WARMED AT 30°C. FOR HALF HOUR	STORED AT ROOM TEMPERATURE (26–28°C.) FOR SIX DAYS, THEN WARMED AT 30°C. FOR HALF HOUR
Time of discharge in seconds....	190.0	414.0	530.0
Viscosity referred to water (30°C.)	2.49	5.41	6.93

1. Viscosity of 2 per cent gelatin at 30°C. after various conditions of storage

Sealed tubes of gelatin, prepared as above, were stored for various periods under different temperature conditions, after which the viscosity at 30°C. was ascertained by observing the time of discharge of a given volume in an Ostwald viscosimeter.[1] The results are shown in table 1. Variations in reading for duplicate tubes were never in excess of two seconds.

It is evident that viscosity at 30°C. of the different batches varied considerably and that it was dependent on the history of

[1] The use of the viscosimeter, in detecting liquefying bacteria, was first suggested by Dr. John C. Torrey in a paper on "The Viscosimeter as an Aid in the Detection of Liquefying Bacteria," Journal of Medical Research, **23**, No. 2, 377–90. 1910.

the gelatin solutions. It was observed that the increase in viscosity of those stored at the higher temperature (26° to 28°C.) was greater than was obtained with those stored in the ice box. The viscosity at 30°C., as was determined in these experiments, is not suitable for comparative studies.

2. Viscosity of 2 per cent gelatin at 40°C. after various conditions of storage

Davis and Oakes, working with unautoclaved gelatin, noted that if the temperature of the solution was not allowed to fall below 40°C. the viscosity (40°C.) remained constant with age. They suggested that if gelatin solutions be heated at 50°C. before making viscosity determinations, and that if the age of the solution be reckoned from the moment it cools to 38.03°C., different workers may readily obtain checks on viscosity measurements. Their experiments extended over relatively short periods (generally not over eight hours and rarely as long as twenty-four hours) and were concerned only with unsterilized gelatin, so that their results are not applicable to bacteriological work without retesting under the conditions encountered in the bacteriological laboratory. The following experiments we believe demonstrate conclusively that sterilized gelatin may be rejuvenated by the method which they suggested even after several weeks of storage.

A 2 per cent gelatin solution, prepared as previously described in sealed tubes, was stored in the ice box at 10 to 12°C. and in thermostats at 22°C., 41°C. and 60°C. The 60°C. series was not begun until after the gelatin had stood at 22°C. for eleven days.

Before making viscosity determinations of the tubes stored at 10°C. and 22°C. they were heated in a water bath at 50°C. for twenty minutes, then placed in a bath at 40°C. and readings begun after ten minutes, which was found to be a more than ample interval for the gelatin to reach the desired temperature. The 41°C. and 60°C. series were not pre-heated before making viscosity readings because these storage temperatures are above the gelation point of gelatin. The results which are given in tables 2 and 2a may be summarized as follows:

TABLE 2

Viscosity of 2 per cent gelatin (Difco) at 40°C. after storage

TEMPERATURES OF STORAGE	PERIOD OF STORAGE					
	2 days	3 days	6 days	10 days	15 days	17 days
	Time of discharge in seconds*					
10–12°C.	127.0	127.0	127.0	127.5	127.0	128.0
22°C.	128.0	127.0	127.0	128.0	128.0	128.0
41°C.	127.5	127.5	127.0	127.0	115.0†	

* Time of discharge immediately after preparation was 128 seconds.
Figures given are an average of 2 or more tubes.
† This determination was checked on 12 tubes.

TABLE 2A

Viscosity of 2 per cent gelatin (Difco) at 40°C. after storage at 22°C. and 60°C.

STORED AT		TIME OF DISCHARGE IN	VISCOSITY (40°C.) REFERRED TO H_2O
22°C.	60°C.		
		seconds	
0	0	128.0	2.19
11 days	2 hours	127.5	2.18
11 days	24 hours	128.0	2.19
11 days	48 hours	125.0	2.14
11 days	72 hours	119.0	2.04
11 days	96 hours	115.0	1.97
11 days	120 hours	104.5	1.79

TABLE 3

Rate of rejuvenation of 2 per cent gelatin (Difco)

TEMPERATURE OF STORAGE	REJUVENATION TEMPERATURE	REJUVENATION TIME				
		5 minutes	10 minutes	15 minutes	20 minutes	40 minutes
		Time of discharge in seconds (at 40°C.)				
10–12°C.	40°C.	131.0	127.5		127.5	127.5
	50°C.	127.5	127.5			
22°C.	40°C.	129.0	129.0		129.0	129.0
	45°C.	129.0	127.5		127.5	127.5
	50°C.	131.0	127.5		127.5	127.5
	55°C.	131.0	127.5	127.5		

1. Autoclaved gelatin stored in sealed tubes for two to seventeen days at 10° or 22°C. retains a constant viscosity when determined at 40°C. after pre-heating at 50°C. for twenty minutes. Gelatin stored at temperatures below the gelation point may therefore be rejuvenated by heating.

2. The viscosity of gelatin (40°C.) stored at 41°C. remained constant for at least ten days. After fifteen days there was a distinct (although not considerable) decrease in viscosity. This is probably due to slow hydrolysis.

3. The viscosity of gelatin (at 40°C.) stored at 60°C. remained unchanged for twenty-four hours after which it slowly decreased. Four days storage at 60°C. effected the same reduction in viscosity as was obtained in fifteen days at 41°C.

3. On the rate of rejuvenation (by heating) of stored gelatin

The viscosity of gelatin at 40°C. was determined after rejuvenation by heating at various temperatures for five to forty minutes. Two series of gelatin tubes, stored in the ice box and the incubator, respectively, for forty-eight hours, were employed. The results, which are given in table 3, show that ten minutes at 40 to 55°C. was ample for rejuvenation.

4. Viscosity of gelatin at various temperatures shortly after heating for rejuvenation

The viscosity of gelatin kept for different periods and at different temperatures was determined, after rejuvenation, at temperatures of 28°, 30°, 32.5 and 40°C. to ascertain whether the viscosity at temperatures below the gelation point would be constant after rejuvenation.

Tubes of gelatin prepared as previously described were stored in the ice box at 10° to 12°C. for four days, then were placed in a 41° incubator for two hours. The tubes were then removed to a water bath at 28°C. and viscosity at that temperature determined after thirty minutes. The time of discharge at 28°C. was one-hundred and seventy five seconds.

Another set of tubes was stored at 26° for three days, then at 41° for one day, after which they were placed in a water bath at 28° for thirty minutes and the viscosity determined. The time of discharge at 28°C. was found to be one hundred and seventy seven seconds.

In another series of observations, two sets of gelatin tubes were stored for three days at 10° and 26°C., respectively, then both sets were kept at 41°C. for three days, after which they were both placed in the ice box over night. The viscosity was then determined at 32.5° and both sets gave a discharge time of one hundred and seventy seconds. These observations indicated that the viscosity of sterilized gelatin at temperatures below the gelation point, if determined at a definite time after rejuvenation, was quite constant irrespective of the history of the gelatin.

TABLE 4

Viscosity of 2 per cent gelatin (Difco), after storage and rejuvenation, at temperatures above and below the gelation point.

STORED AT 22°C.	VISCOSITY* (TIME OF DISCHARGE IN SECONDS)	
	40°C.	30°C.
0†	128.0	155.0
1 day	128.0	155.0
2 days	129.0	154.0
3 days	129.0	153.5
4 days	129.0	154.0
5 days	129.0	154.0
6 days	129.0	154.0
7 days	129.0	154.0

* Rejuvenated by heating at 50°C. for fifteen minutes and kept at viscosity temperature for fifteen to twenty minutes before reading.

† Immediately after preparation.

Finally a series of viscosity determinations (after rejuvenation by heating) were made at 40°C. and at 30°C. on gelatin stored at 22°C. for one to seven days. The results are tabulated in table 4 below.

It is concluded from the results obtained that the viscosity of gelatin at temperatures below the gelation point, as well as at 40°C., may be rendered sufficiently constant for bacteriological work by heat rejuvenation.

B. THE EFFECT OF BACTERIAL GROWTH ON VISCOSITY AND FORMOL TITRATION

1. Observations with mesophilic organisms

A 2 per cent gelatin solution, prepared as described above, was distributed in Blake bottles, and autoclaved at 15 pounds for fifteen minutes. Each of the bacteria tested was then inoculated into three bottles and, after storage for one, two and four days at

TABLE 5

Effect of period of incubation on formol (Sorensen) titration and viscosity after rejuvenation of 2 per cent gelatin (Difco)

INCUBATION (26°C.) IN DAYS	CONTROL	PS. FYOCTANEA	PS. FLUORESCENS	BACT. VULGARIS	ERYTHRO PRODIGIOSUS	BACT. AEROGENES
	Time of discharge in seconds					
0	138	138	138	138	138	138
1		134	131	136	80	138
2		87	87	89	72	138
4	138	76	75	76	72	138
	Viscosity (40°C.) referred to water					
0	2.36	2.36	2.36	2.36	2.36	2.36
1		2.29	2.24	2.33	1.37	2.36
2		1.49	1.49	1.52	1.23	2.36
4	2.36	1.30	1.28	1.30	1.23	2.36
	Increase in formol titration (cc. N/1 NaOH per 100 cc. medium)					
0						
1		0.4	0.4	0.4	0.4	0
2		2.4	0.4	0.4	2.8	0.8
4	0	3.8	4.0	6.2	17.6	0.8

26°C., the change in viscosity and formol titrations was ascertained. Viscosity readings were made at 40°C., the gelatin first being heated at 50°C. for twenty minutes. Formol titrations are expressed in terms of N/1 NaOH required for 100 cc. of the medium for neutralization after treatment with formaldehyde.

The non-liquefying organism *Bact. aerogenes* showed no change in viscosity and practically no increase in the formol titration. With the other organisms studied, it is evident that the change

in viscosity could be detected before there was any appreciable change in formol titration.

The *Erythrobacillus prodigiosus* almost completely liquefied the gelatin before the titration figure began to rise at all and this liquefaction was extremely rapid compared to the other organisms With respect to the formol titration, the liquefying organisms again fall into two groups; those in which the rise is rapid and considerable (*E. prodigiosus*) and those in which the increase is comparatively less marked.

2. Observations with thermophilic organisms

To ascertain whether the change in viscosity and formol titration may be utilized to detect proteolysis by thermophiles, the following experiment was performed.

The medium employed consisted of 10 per cent Difco gelatin and 0.1 per cent pepton (Difco) neutral to phenolphthalein. Twenty-five cubic centimeter portions were placed in large test tubes and sterilized at 15 pounds for fifteen minutes. Four thermophilic organisms, isolated from soil and corn meal, were inoculated into a series of tubes and incubated at 55°C. One tube of each organism was removed daily for four days for determination of viscosity, which was made at 55°C., and formol titration. The results are shown in table 6.

It is apparent from the table that at a temperature of 55°C. gelatin is slowly hydrolized. Its relative viscosity was unchanged in two days but dropped from 12.1 to 11.4 on the third day and to 10.0 on the fourth day. The formol titration of uninoculated gelatin rose but very slightly, the increase being but 1 cc. in four days. All of the organisms employed were gelatin liquefiers. Here also it will be seen (if the change in viscosity and the increase in formol titration are compared) that gelatin liquefaction may be detected by observing the decrease, in viscosity, about twenty-four hours before there is any appreciable increase in the formol titration. After twenty-four hours incubation all tubes solidified on storage over night at 22°C., whereas the viscosity in the inoculated cultures had decreased considerably so that the measurement

of change in viscosity is much more delicate than the old method of ascertaining whether the medium would solidify on cooling. The change in viscosity and formol titration is applicable to the study of gelatin decomposition by thermophiles.

TABLE 6

Showing change in viscosity (55°C.) and formol titration effected by thermophiles in 10 per cent gelatin (Difco) at 55°C.

INCUBATION DAYS AT 55°C.	CONTROL	NO. 5	NO. 17	NO. 48	NO. 61
	Time of discharge in seconds				
0	624	624	624	624	624
1	624	300	452	438	396
2	624	156	244	396	164
3	588	120	125	203	138
4	540	115	116	131	117
	Viscosity (55°C.) referred to water				
0	12.1	12.1	12.1	12.1	12.1
1*	12.1*	5.55*	8.35*	8.10*	7.31*
2	12.1*	2.88	4.33	7.33	3.03
3	11.4*	2.22	2.31	3.76	2.55
4	10.0*	2.12	2.14	2.42	2.16
	Formol titration (cc. N/1 NaOH per 100 cc.)				
0	9.20	9.20	9.20	9.20	9.20
1	9.20	10.10	9.20	9.10	9.70
2	9.30	14.85	10.00	10.80	13.40
3	9.65	22.00	21.80	13.00	19.20
4	10.20	27.90	29.00	23.00	22.90
	Increase in formol titration over control				
1		0.90	0.0		0.50
2		5.55	2.70	1.50	4.10
3		12.35	12.15	3.35	9.65
4		17.70	19.70	12.80	12.70

* Solidified on standing over night at 22°C.

SUMMARY

The results of this study may be summarized briefly as follows: Viscosity of 2 per cent Difco gelatin, stored at temperatures of 10 to 22°C. for two to seventeen days, may be rendered constant

by preheating the gelatin at 50°C. before making the viscosity reading.

Gelatin stored at 41°C. retains a constant viscosity at that temperature for at least ten days, after which it decreases slowly.

The viscosity of gelatin at temperatures below the gelatin point is rendered sufficiently constant for bacteriological work by preheating the gelatin at 41 to 50°C.

The changes in viscosity and formol titration were found convenient methods for detecting proteolysis by mesophilic and thermophilic bacteria.

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HYDROGEN SULFIDE DETERMINATION IN BACTERIAL CULTURES AND IN CERTAIN CANNED FOODS

C. R. FELLERS, O. E. SHOSTROM AND E. D. CLARK

Laboratories of the Northwest Branch, National Canners Association, Seattle, Washington

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The determination of hydrogen sulfide in bacterial cultures has always been regarded as unsatisfactory. The recent Manual of Methods for pure culture study prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists (1923) gives a provisional method for observing hydrogen sulfide production only and states that certain weaknesses in the methods have been pointed out. Use is made of the lead acetate agar method as recommended by Burnet and Weissenbach (1915), Maymone (1917), Jordan and Victorson (1917), Kligler (1917) and Thompson (1921). Briefly the medium consists of a beef extract agar containing 30 grams peptone and 5 cc. of a 0.1 per cent basic lead acetate solution per liter (pH 7.2 to 7.6). An organism producing hydrogen sulfide when inoculated into this medium at a suitable temperature causes blackening of the medium. In general the more marked the intensity of blackening the greater the amount of hydrogen sulfide present. Obviously this procedure yields only qualitative results.

The only other attempt at even an approximate determination of hydrogen sulfide in bacterial cultures is the method used by Schardinger (1894), Dunham (1897), Redfield (1912), Myers (1920) and Tilley (1922). These investigators attempted to correlate hydrogen sulfide formation by water bacteria with the sanitary condition of the water.

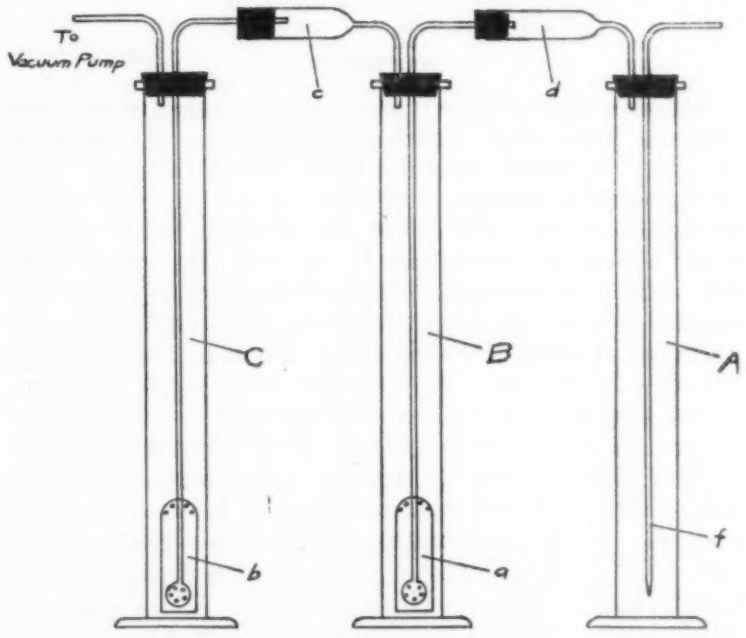
The technic of this method is as follows: A strip of filter paper moistened with a 10 per cent solution of neutral lead acetate is suspended in the mouth of a flask in such a manner that a given length is exposed to the action of any hydrogen sulfide formed. After incubation the approximate number of millimeters of lead acetate paper blackened is used as the basis of comparison. Obviously this method gives only approximate quantitative results.

In gas analysis however several quantitative methods have been used. Hydrogen sulfide may be quantitatively determined by Dupasquier's method, a measured quantity of gas being drawn through a solution of iodine to which some starch paste has been added. This method has also been used and improved upon by McBride and Weaver (1913) of the United States Bureau of Standards. They found that the volatility of the iodine introduced an error in this determination which could be obviated by absorbing the volatilized iodine in sodium thiosulfate. This improved method is somewhat like the one presented in this paper. Another method that has had considerable use is the passing of a measured quantity of gas through bromine water and determining the sulfur gravimetrically. This method is slow and the bromine is disagreeable to work with. There are other ways, such as the Fresenius gravimetric method, suggested for the determination of hydrogen sulfide in gas but these need not be mentioned here.

The advantages of a rapid volumetric method are obvious. Quantitative methods in bacteriology, particularly in pure culture studies, have been much neglected. Except as a qualitative test the lead acetate agar and paper strip methods must be considered inadequate and inaccurate. It is felt that any addition to quantitative methods along these lines is particularly welcome. With such a purpose in mind the method given in this paper for a rapid volumetric determination of hydrogen sulfide formed in bacterial cultures was developed. The problem of liberating the hydrogen sulfide from the solutions without splitting off any sulfur was the main difficulty to overcome. This was finally accomplished by an aeration

method. The liberated hydrogen sulfide was passed through standard iodine solution, the volatilized iodine collected in standard sodium thiosulfate solution, and finally the two solutions titrated with iodine or thiosulfate, depending upon which was present in excess.

Fig. I



APPARATUS

The apparatus employed is illustrated in figure 1; *A*, *B* and *C* are cylinders containing the sample, standard iodine and sodium thiosulfate solutions respectively; *a* and *b* are Folin absorption tubes; *c* and *d* are Folin drying tubes to prevent any spray from being carried over into cylinders *B* and *C*, or into the tube leading to the vacuum pump. A glass tube *f* with a small opening is used to cause considerable agitation

in the sample. Another cylinder may be used containing potassium hydroxide solution to absorb any hydrogen sulfide that might pass in along with the air from the laboratory. In our laboratory this was found to be unnecessary. It was also recognized that cork or rubber stoppers absorb small amounts of iodine, but the iodine solution was so weak and so little came in contact with the corks that no measurable error was introduced by the use of cork stoppers.

PROCEDURE

Introduce into cylinder *B* an excess of standard N/40 iodine solution. Then place sufficient N/40 sodium thiosulfate in cylinder *C* to absorb all of the iodine from the iodine solution during aeration (usually 10 or 15 cc. is ample). Add enough water to these two cylinders so that the level stands about 4 inches above the upper holes in the Folin absorption tubes. Into cylinder *A* introduce 100 cc. of the solution, culture, or suspension to be analyzed and acidify with phosphoric acid. An excess of phosphoric acid will do no harm. If the sample is known to contain a large amount of hydrogen sulfide an aliquot may be used, thereby eliminating the use of too great an amount of the iodine solution. Place connecting tubes in position at once, pass air through the apparatus slowly for two or three minutes, then more rapidly for at least fifteen minutes. The final rate and time of aeration depend upon the amount of frothing. By the addition of a drop or two of caprylic alcohol frothing can be prevented. Under most conditions fifteen minutes is sufficient to remove all of the hydrogen sulfide. The two solutions in *B* and *C* are then mixed, cylinders and bulbs being carefully washed to remove all of the reagents, and titrated with either iodine or sodium thiosulfate solutions, depending upon which is in excess, using starch as an indicator and making the necessary correction for the blank. The results are calculated as percentage of hydrogen sulfide, as milligrams per 100 cc. of sample, or as parts per million.

EXPERIMENTAL

Volatility and recovery of iodine by aeration

Since it was found, in preliminary experiments using only one cylinder, that a considerable error was caused by loss of iodine from the standard iodine solution, due to volatilization, a second cylinder containing standard sodium thiosulfate solution was used to absorb the iodine volatilized. By using this method of recovery a larger excess of standard iodine solution could be used.

To determine the amount of the error due to volatilization of iodine from its solution by aeration, known amounts of standard iodine and standard sodium thiosulfate were intro-

TABLE 1

Volatility and recovery of iodine by aeration

CYLINDER B	CYLINDER C						
N/40 iodine added (1)	N/40 thiosulfate used to titrate excess iodine (2)	N/40 sodium thiosulfate added (3)	N/40 iodine used to titrate excess thiosulfate (4)	N/40 iodine volatilized (1)-(2)	N/40 iodine recovered (3)-(4)	Iodine volatilized	Volatilized iodine recovered
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*
6.61	3.46	8.05	4.93	3.15	3.12	47.65	99.47
7.72	4.76	6.68	3.76	2.96	2.92	38.34	98.65
9.20	3.79	9.96	4.59	5.41	5.35	58.80	98.89

duced into cylinders *B* and *C* respectively, and air was drawn through the apparatus by means of a vacuum pump. After aerating for ten minutes the known solutions were titrated to determine the amount of iodine volatilized and recovered. The loss due to volatilization during aeration is large but inasmuch as the recovery is practically complete no appreciable error is introduced.

The data in table 1 represent only a few actual determinations taken at random, whereas a great number were made using varying amounts of iodine and thiosulfate as well as varying the time and speed of aeration. The results show that within a reasonable experimental error, the iodine recovery is complete.

Time required for the liberation of hydrogen sulfide from solutions by aeration

The length of time required for complete liberation of the hydrogen sulfide was determined by adding known amounts of a water solution of hydrogen sulfide, aerating for various periods of time, titrating the added iodine and sodium thiosulfate and also the hydrogen sulfide remaining in the solution. Table 2 gives one series of results out of the several made showing that fifteen minutes is a sufficient time for the liberation of the hydrogen sulfide.

TABLE 2

Time required for the liberation of hydrogen sulfide from solutions or cultures

TIME OF AERATION MINUTES	N/40 IODINE ADDED	N/40 $Na_2S_2O_3$ USED	IODINE N/40 USED BY THE H_2S	H_2S RECOVERED	H_2S ADDED	H_2S REMAINING AFTER AERATION	H_2S RECOVERED
	cc.	*cc.*	*cc.*	*mgm.*	*mgm.*	*mgm.*	*per cent*
1	23.79	19.63	4.16	13.28	16.80	3.07	79.04
2	22.91	18.41	4.50	14.26	17.11	2.63	83.34
3	21.53	16.31	5.21	16.51	17.33	1.29	95.84
4	22.13	16.85	5.28	16.73	17.56	1.10	94.78
5	20.86	15.44	5.42	17.18	17.84	1.01	96.30
6	21.45	15.86	5.59	17.72	17.91	0.69	98.94
8	22.37	16.62	5.75	18.22	18.38	0.38	99.13
10	20.90	15.01	5.89	18.67	18.60	0.16	100.37
12	21.51	15.65	5.86	18.57	18.76	0.37	98.98
14	23.20	17.20	6.00	19.02	18.86	0.16	100.84
16	20.70	14.73	5.97	18.92	18.86	0.34	100.31
16	27.06	10.55	16.51	52.33	52.68	0.25	99.33
16	25.76	9.40	16.36	51.86	52.36	0.12	99.04
16	16.20	8.20	8.00	25.36	25.58	0.06	99.14
16	17 00	8.60	8.00	25.36	25.58	0.19	99.14

Effect of certain bacterial decomposition products on the liberation and recovery of hydrogen sulfide from solutions

Dunham's solution, together with known decomposition products, were mixed with hydrogen sulfide solution and aerated for fifteen minutes to determine if they would retard or interfere with the volatility of the hydrogen sulfide. In table 3 it will be seen that neither the media nor the known decomposition products have any noticeable effect on the liberation

or recovery of hydrogen sulfide, excepting large amounts of ammonia, and this of course is overcome by addition of acid. Large amounts of ammonia are however, seldom present in cultures or foods where this method is applicable.

TABLE 3

Effect of media and certain decomposition products upon the liberation and recovery of hydrogen sulfide from solutions

DECOMPOSITION PRODUCTS ADDED	MEDIA ADDED 2.5 PER CENT PEPTONE	n/40 IODINE ADDED	n/40 $Na_2S_2O_3$ USED	n/40 IODINE USED BY THE H_2S	H_2S RECOVERED	H_2S ADDED	IODINE RECOVERED	H_2S RECOVERED
	cc.	*cc.*	*cc.*	*cc.*	*mgm.*	*mgm.*	*per cent*	*per cent*
	50	15.49	15.49	0	0	0	100.00	
	50	11.54	11.51	0	0	0	99.74	
	50	11.48	11.44	0	0	0	99.65	
	50	12.83	12.81	0	0	0	99.79	
Formic*, acetic, butyric, propionic, valeric acids, phenol, indol, skatol, alcohol, NH_4OH and NH_4Cl	50	14.24	14.19	0	0	0	99.64	
	50	11.83	11.74	0	0	0	99.24	
	50	12.73	12.68	0	0	0	99.61	
	50	12.93	12.87	0	0	0	99.49	
	50	17.43	9.02	8.01	25.36	25.58		99.14
	50	25.92	9.65	15.87	50.30	50.72		99.17
	50	49.35	7.82	41.23	130.69	130.92		99.82
	50	30.90	9.16	21.70	68.78	60.07		99.37
2 cc. of 30 per cent NH_4OH	50	19.98	8.75	11.23	35.59	28.21		128.1
	50	17.32	6.69	10.63	33.69	28.21		119.4
	50	18.75	7.61	11.14	35.30	28.21		125.1

* One-fourth cubic centimeter each of the acids and NH_4OH, 0.5 cc. alcohol and 0.1 gram of each phenol, indol, NH_4Cl and skatol were used.

Effect of acids and alkalies on the liberation of hydrogen sulfide from solutions

Dunham's Solution containing known amounts of hydrogen sulfide was treated with potassium hydroxide, aerated and titrated to determine the amount of iodine used up by the hydrogen sulfide. The same sample was then treated with

sufficient phosphoric acid (H_3PO_4) to make it distinctly acid and again aerated and titrated. Tabular results given in table 4

TABLE 4

Effect of acids and alkalies on the liberation of hydrogen sulfide from solutions

KOH OR H_3PO_4 ADDED	MEDIA ADDED 50 CC.	N/40 IODINE ADDED	N/40 $NO_2S_2O_3$ USED BY THE H_2S	IODINE USED UP BY THE H_2S	H_2S RECOVERED	H_2S ADDED	H_2S RECOVERED
	Series 1						
		cc.	*cc.*	*cc.*	*mgm.*	*mgm.*	*per cent*
KOH	Dunham's solution with 25 grams peptone	11.82	10.75	1.07	3.39	26.74	12.65
H_3PO_4	Dunham's solution with 25 grams peptone	18.61	11.35	7.26	23.01	26.74	86.05
Total				8.33	26.40	26.74	98.7
	Series 2						
KOH	Dunham's solution with 25 grams peptone	11.17	10.45	0.72	2.28	26.54	8.59
H_3PO_4	Dunham's solution with 25 grams peptone	18.03	10.45	7.58	24.02	26.54	90.12
Total				8.30	26.30	26.54	98.71
	Series 3						
KOH	Dunham's solution with 25 grams peptone	10.64	10.11	0.53	1.68	28.21	5.95
H_3PO_4	Dunham's solution with 25 grams peptone	18.56	10.24	8.32	26.37	28.21	93.48
Total				8.85	28.05	28.21	99.42

Peptone solutions and canned salmon when treated with hot 10 per cent potassium hydroxide solution liberated volatile sufur, which could be recovered by acidifying the solution and aerating as described. Due to the slowness of this reaction and the gradual hydrolysis of proteins and intermediate products, no definite endpoint is reached and the hydrogen sulfide recovered after acidifying and aerating is in no sense an indication of decomposition.

show that potassium hydroxide combines with the hydrogen sulfide and is finally liberated by acid treatment showing

TABLE 5

Milligrams of hydrogen sulfide produced by various bacteria in 100 cc. of 2.5 per cent "Difco" peptone media

ORGANISM	TIME OF INCUBATION		
	Forty-eight hours	Ninety-six hours	One hundred forty-four hours
Bact. typhi (Hopkins)	0	0	3.54
Bact. paratyphosum A, strain I	0	0	0
Bact. paratyphosum A (Weinzirl)	0	0	0
Bact. paratyphosum B (Cascade)	5.34	5.84	8.48
Bact. paratyphosum B (Rowland)	6.42	5.30	5.56
Bact. paratyphosum B (Jordan)	6.30	6.08	7.20
Bact. enteritidis, strain I	2.70	3.45	4.66
Bact. enteritidis, strain II	2.86	7.40	7.90
Bact. dysenteriae (Flexner)	0	0	0
Bact. dysenteriae (Shiga)	0	0	0
Bact. suipestifer	0	0	0
Human feces	10.20	19.12	21.22
Bact. coli	14.06	12.18	13.74
Bact. cummunior	16.04	12.16	16.44
Bact. cloacae	0	0	1.32
Bact. aerogenes	0	0	0
Bact. lactis-viscosus	0	0	0
Bact. alkaligines	0	0	0
Bact. acidi-lactici	0	0	0
P. vulgaris	6.64	6.92	12.04
Bact. capsulatum	0	0	0
Msp. comma	0	0	0
B. mycoides	0	0	0
B. mesentericus	0	0	0
B. megatherium	0	0	0
B. thermoidifferens	0	0	0
B. centrosporus	0	0	0
Thermophile from canned corn	0	0	0
B. aerothermophilus	0	0	0
B. subtilis	0	0	0
B. albolactus	0	0	0
B. flexus	0	0	0
B. cohaerens	0	0	0
B. terminalis	0	0	0
B. vulgatus	0	0	0
B. circulans	0	0	0
B. cereus	0	0	0
B. mesentericus flavus	0	0	0
B. pseudo-tetanicus	0	0	0

TABLE 5—*Continued*

ORGANISM	TIME OF INCUBATION		
	Forty-eight hours	Ninety-six hours	One hundred forty-four hours
Skatol liberating clostridium (Fellers)	1.64	33.72	34.54
Ps. flurorescens	0	0	0
Bact. aurescens	0	0	0
Erw. carotovora	0	0	0
Thiothrix janiculatus	0	0	0
Erythrob. prodigiosus	0	0	0
Mic. albus	0	0	0
Sar. lactis-acidi	0	0	0
Sar. ventriculi	0	0	0
Sar. lutea	0	0	0
Staph. albus	0	0	0
Sacch. ellipsoideus	0	0	0
Asperigillus flavus	0	0	0
Penicillium sp?	0	0	0
Torula from clams	0	0	0

the necessity of an acid reaction. Due to the presence of free hydrogen sulfide in the cylinder above the solution, the potassium hydroxide did not combine with the total amount of hydrogen sulfide added. The small amount remaining unfixed by the alkali was absorbed by the iodine solution during aeration and accounts for the small per cent recovered before addition of the acid.

Quantitative determination of hydrogen sulfide produced by various bacteria, yeasts and molds in Dunham's solution containing 2.5 per cent "Difco" peptone

Various bacteria, yeasts and molds were inoculated into 300 cc. of Dunham's media using "Difco" peptone and incubated at 37°C. for one hundred and forty-four hours. Aliquots of 100 cc. were withdrawn at forty-eight hour intervals by means of sterile pipettes, transferred to the apparatus shown in figure 1, acidified, aerated and the amount of hydrogen sulfide liberated determined by titration as described under determination.

Witte's and Armours' peptone were also used but proved unsatisfactory because upon aerating media before inoculation a considerable reduction of the iodine took place. Sterilization of the media for fifteen minutes at 10 pounds pressure in an autoclave liberated hydrogen sulfide from Witte's and Armours' peptone but not from Difco.

Table 5 needs but little comment. All strains of *Bact. paratyphosum* B produced hydrogen sulfide, while none of the strains of *Bact. paratyphosum* A and only a few of the common

TABLE 6

Comparison of commercial peptones for hydrogen sulfide liberation by bacteria

	ARMOUR'S PEPTONE			WITTE'S PEPTONE			DIFCO PEPTONE		
	Forty-eight hours	Ninety-six hours	One hundred forty-four hours	Forty-eight hours	Ninety-six hours	One hundred forty-four hours	Forty-eight hours	Ninety-six hours	One hundred forty-four hours
Feces	16.86	29.46	47.54	32.12	5.70	4.75	10.20	19.12	21.22
Bact. cloacae	0	5.04	25.86	23.16	7.40	0.00	0	0	1.32
Bact. aerogenes	0	6.06	32.84	12.62	0.00	0.00	0	0	0
B. mycoides	0	0	33.32	11.68	8.64	0.00	0	0	0
Bact. enteritidis	45.56	Lost	40.56				2.70	3.45	4.66
Bact. coli	16.86	29.46	47.54	33.28	16.58	3.12	14.06	12.18	13.74
Bact. communior				10.00	0.00	0.00	16.04	12.16	16.44
Skatol liberating clostridium	25.08	19.08	14.0	32.7	29.28	22.58	1.64	33.72	34.54
Bact. typhosum	24.85	13.20	23.98				21.71	16.00	29.48

organisms were able to produce any. Yeasts and molds all gave negative amounts. A skatol liberating clostridium (Fellers) gave a very large amount of hydrogen sulfide. None of the 17 species of sporeforming aerobic bacteria produced hydrogen sulfide from Difco peptone. Likewise the non-proteolytic cocci and non-sporing rods did not possess this ability. The colon-typhoid and proteus groups together with the proteolytic anaerobes are the organisms capable of liberating hydrogen sulfide from "Difco" peptone. The use of this test in

studying the biochemical properties of microörganisms should aid materially in their identification and classification.

Both Armour's and Witte's peptone were used for purposes of comparison with Difco peptone but proved to be unsatisfactory. This was particularly true in the case of Witte's peptone. Both these brands when heated in 2.5 per cent concentration in an autoclave at 10 pounds pressure for ten minutes produced considerable hydrogen sulfide. Witte's peptone was more unstable in this respect than Armour's. Media containing either Armour's or Witte's peptone when sterilized, aerated and titrated with the standard iodine thiosulfate solution were found to neutralize considerable iodine, the amount varying greatly in several tests. A pink color was often present which interfered with the titration. Media containing these two brands of peptone and inoculated with bacteria gave discordant results in several tests.

As a result of this work the authors believe "Difco" peptone gives most reliable results, followed by Armour's. Witte's peptone is not suitable for quantitative hydrogen sulfide determination.

Quantitative determination of hydrogen sulfide in canned salmon and other canned foods

Salmon canned at various stages of decomposition, normal commercial canned salmon, swelled and normal cans of other food products, were treated the same as the bacterial cultures to determine their hydrogen sulfide content.

The method was found to be entirely applicable to the determination of hydrogen sulfide in various food products. Preliminary experiments showed satisfactory recoveries of hydrogen sulfide from salmon, gooseberries and shrimp, which had been artificially impregnated with known amounts of the gas. It was thought that some hydrogen sulfide might be formed during the decomposition of salmon or shrimp flesh but none was found either in the experimental or commercial samples examined. It is entirely probable however that where

certain hydrogen sulfide liberating bacteria are present in decomposed foods, hydrogen sulfide would be found. The authors believe that its presence in most cases indicates decomposition.

It is well known that certain acid fruits like gooseberries, cherries, apples and rhubarb when canned readily corrode the container, giving rise under favorable conditions to the forma-

TABLE 7

Hydrogen sulfide content of certain normal and abnormal canned foods

DESCRIPTION OF SAMPLE	ODOR	H_2S PER KILO OF SAMPLE
		mgm.
Red salmon one day old before canning	Normal	0
Red salmon two days old before canning	Normal	0
Red salmon three days old before canning	Stale	0
Red salmon four days old before canning	Slightly tainted	0
Red salmon five days old before canning	Tainted	0
Red salmon six days old before canning	Putrid	0
Red salmon (normal)	Normal	0
Chinook salmon (normal)	Normal	0
Coho salmon (normal)	Normal	0
Pink salmon (normal)	Normal	0
Chum salmon (normal)	Normal	0
Shrimp (swelled)	Slightly tainted	0
Shrimp (normal)	Normal	0
Gooseberries (swelled)	Hydrogen sulfide	366.0
Gooseberries (swelled)	Hydrogen sulfide	90.0
Gooseberries (swelled)	Hydrogen sulfide	81.0
Gooseberries (swelled)	Hydrogen sulfide	61.0
Gooseberries (normal)	Normal	0
Gooseberries (normal)	Normal	0
Gooseberries (normal)	Normal	0
Gooseberries (normal)	Normal	0
Gooseberries (normal)	Normal	0

tion of hydrogen gas. Where easily reducible sulfur compounds are present hydrogen sulfide may also be formed. This is undoubtedly what occurred in the canned gooseberries showing hydrogen sulfide reported in table 7. These samples had all been sprayed shortly before canning with a lime-sulfur mixture to prevent mildew. Normal gooseberries contained no volatile sulfur.

SUMMARY

The method as described is simple, rapid, easily executed, and reliable for the quantitative determination of the hydrogen sulfide formed in bacterial cultures and in canned foods containing free hydrogen sulfide. The hydrogen sulfide volatilized from acid solutions with air, is drawn through standard iodine; the volatilized iodine absorbed by passing through standard sodium thiosulfate; and the hydrogen sulfide determined by titration. Care must be exercised in washing the cylinders and bulbs after aeration and correction applied for starch indicator used. Standard apparatus is used throughout and no large quantities of reagents required. Fifteen minutes aeration of the culture or suspension being tested was sufficient to recover from 99 to 100 per cent of the hydrogen sulfide present.

Ordinary bacterial decomposition products such as volatile fatty acids, phenols, indol, skatol, alcohol and small quantities of ammonia did not affect the liberation of hydrogen sulfide from cultures or food suspensions. Twelve out of 53 microorganisms tested as to their hydrogen sulfide liberating properties in "Difco" peptone gave positive results. Armour's and Witte's peptone gave inconsistent results and in general proved unsatisfactory for this work. In general the non-proteolytic types of bacteria including the cocci, aerobic sporing rods, yeasts and inactive water and soil bacteria fail to liberate hydrogen sulfide from proteins. Certain members of the colon and proteus groups, *Bact. paratyphoid* B, *Bact. enteritidis* and a proteolytic anaerobe produced the greatest amounts of hydrogen sulfide.

The method may be used satisfactorily to determine volatile sulfur in foods. Canned gooseberries which had been sprayed while immature with lime-sulfur spray, contained large quantities of hydrogen sulfide. Under the conditions of the experiment no hydrogen sulfide was liberated from decomposed salmon or shrimp.

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NITRIFICATION AND THE NITRIFYING ORGANISMS. I[1]

R. NAGAN GOWDA[2]

Iowa State College

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Since 1890 when Winogradsky (1890) announced the isolation of two organisms which produce nitrates from ammonia, numerous investigators have attempted to repeat his work. A few have claimed the isolation of organisms that form nitrites and nitrates and also conform to the requirement of purity of the cultures laid down by Winogradsky, that they should not grow in alkaline bouillon. No one, however, has been successful in duplicating the work of Winogradsky in its entirety and the majority have absolutely failed to secure cultures of organisms which would meet Winogradsky's criterion of purity.

The whole problem of the isolation of the nitrifiers and the methods of such isolation therefore still remain to be solved.

The literature on the subject is very extensive. Perroti (1905) reviewed the work that was done previous to 1905 and recently Gibbs (1919) has published a detailed review of the literature up to 1919. Since then Fred and Davenport (1921) have reported on the isolation of *Nitrobacter* and its tolerance towards certain kinds of organic matter.

Only a few investigators have reported on the isolation and description of organisms that are encountered in the purification of nitrifiers. Berstyn (1903) found in his nitrobacter culture of F_{20} generation *Bac. comes*, *Bac. modestum*, *Bac. debile* and *Pseu-*

[1] Part of a thesis submitted to the faculty of the Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] The author is indebted to Dr. P. E. Brown for his useful criticism and for reading the manuscript, to Dr. Paul Emerson for his help during the progress of the work and in the interpretation of results, to Dr. R. E. Buchanan and Dr. Max Levine for their many valuable suggestions, and to Dr. H. W. Johnson for designing the micropipette holder used in this work.

domonas humicola. Beijerinck (1914) found in his nitrobacter cultures, *Bacillus nitroxus*. Gibbs observed in his cultures of *Nitrosomonas* and *Nitrobacter*, three organisms, namely a yellow pigment forming bacillus, a minute bacillus and a small coccus.

Beijerinck also found in his *Nitrobacter* cultures, *A. robur* Krainsky, *A. griseus* Krainsky and occasionally *A. diastaticus* Krainsky and *A. cellulosae* Krainsky. The Hypomicrobium of Stutzer and Hartleb (1901) reminds one of the actinomyces.

EXPERIMENTAL

The purpose of this investigation was to isolate in pure culture, if possible, the nitrite and the nitrate formers.

Isolation of the nitrite formers

Subculture method. Gibbs' (1919) nutrient solution modified, was employed:

Solution 1

$(NH_4)_2SO_4$	1.0 gram
K_2HPO_4	1.0 gram
NaCl	2.0 grams
$MgSO_4$	0.5 gram
$Fe_2(SO_4)_3$	Trace
Water (conductivity)	1000 cc.

Solution 2

$MgCO_3$	5.0 grams
Water (conductivity)	100 cc.

Five cubic centimeters of solution 1 with 0.5 cc. of solution 2 were placed in flasks and inoculated with soil. Nitrites were first found after five to six days, by Trommsdorf's reagents. Subcultures took about fifteen days to show oxidation. Further subcultures were then carried through six generations. Complete oxidation of the $(NH_4)_2SO_4$ did not occur in cultures eight weeks old.

Liquid cultures of the sixth generation were not pure according to Winogradsky's test in bouillon and subcultures of the tenth generation were also impure by this test.

Washed agar. Agar soaked in water eight to ten days was used with various combinations of nutrient salts as shown in table 1. Tests were made with all the media except, nos. 3 and 9

TABLE 1*

Media used

SOLUTION NUMBER	a	b			c		HOW STERILIZED	AMOUNT USED PER PLATE	pH WITH 10 CC. WASHED AGAR
	KH_2PO_4	$(NH_4)_2SO_4$	$MgSO_4$	$Fe_2(SO_4)_3$	NaCl	Na_2CO_3			
	gm.	gm.	gm.	gm.	gm.	gm.			
3†	1.5	1.5	0.75	0.02	3.0	1.5	a, b, c, separately	1 cc. each of a, b, c	8.6
4	1.5	1.5	0.75	0.02	3.0	$MgCO_3$ excess	a, b, c, separately	1 cc. each of a, b, c	8.8
5	1.5	1.5	0.75	0.02	3.0	$CaCO_3$ excess	a, b, c, separately	1 cc. each of a, b, c	8.2
6	1.5	1.5	0.75	0.02	$CaCO_3$ excess	$CaCO_3$ excess	a, b, c, separately	1 cc. each of a, b, c	
7	1.5	1.5	0.75	0.02	NaCl 3.0	$MgCO_3$+ $CaCO_3$	a, b, c, separately	1 cc. each of a, b, c	8.0
8a	1.5	1.5	0.75	0.02	NaCl 3.0	Na_2CO_3 1.5	a, b, c, sterilized together	3 cc. sterilized mixture	7.6
8b	1.5	1.5	0.75	0.02	NaCl 3.0	$MgCO_3$ excess	a, b, c, sterilized together	3 cc. sterilized mixture	7.6
8c	1.5	1.5	0.75	0.02	NaCl 3.0	$CaCO_3$ excess	a, b, c, sterilized together	3 cc. sterilized mixture	7.6
9	15.0	15.0	7.50	0.20	NaCl 30.0	Na_2CO_3 15.0	a, b, c, sterilized separately	0.1 cc. each of a, b, c	
10	15.0	15.0	7.50	0.20	NaCl 30.0	$NaCO_3$ 15.0	a, b, c sterilized together	0.3 cc. of the mixture	

* a, b and c were each dissolved in 100 cc. of conductivity water. The reaction was determined by the color standards of Medalia (1920), (1922).

† This solution suggested by Skinner and Reid (1921).

and growth occurred on all the plates. Nitrites were found in all tests but all cultures isolated from the colonies showed impurity by the bouillon test.

Two types of colonies grew on these plates, type 1, deep, circular, small and pale blue with well defined edges; and type 2, surface spreading and almost colorless. Both types were inoculated on agar plates with no. 8b and also into solution cultures with nos. 1 and 2, which had air bubbled through them. Growth occurred in all cultures and positive tests for nitrites were secured but the bouillon test showed impurity.

Silicic acid gel. This medium was made up much as Gibbs (1919) prepared it. In dialyzing the silicic acid, a bell jar with a membrane at the bottom and an opening at the top was used, to free the air of CO_2. A 2.5 per cent silicic acid solidified readily with solutions 9 and 10. Beijerinck's (1914) method of pouring into dishes equivalent solutions of HC1 and Na_2SiO_3 was also used in part of the work.

In a series of seven experiments in which the inoculum was obtained from liquid cultures made from plates, growth was noted in four to twenty days and three types of colonies were observed. Type 5 appearing first, was irregular with a rough, somewhat flat surface. It was slightly yellow in color, with an erose edge and an amorphous internal structure. The colony was about 0.5 mm. in diameter. The organism was a coccus about 0.4 to 0.5 μ in diameter. Type 4 was a small, circular colony about 50 μ in diameter, greenish-yellow in color, with an amorphous internal structure. The organism was a coccus. Type 3 was yellow in color and about 200 μ in diameter. It had a regular outline, almost circular, a rough elevated surface and a finely granular internal structure. The organism was a coccus, from 1.5 to 1.0 μ in size. All three types gave tests for nitrites but all grew in bouillon.

In another series of 13 experiments, type 3 gave a positive test for nitrites in silica plates while nos. 4 and 5 gave negative tests. No. 3 did not grow on nutrient agar plates. Colonies of no. 3 were again inoculated on silica plates but gave negative tests for nitrites. Tests of the three types of colonies, in liquid cultures, tested every week for eight weeks, showed no oxidation for nos. 4 and 5 and nitrites were shown only in 10 per cent of the tests with no. 3. Growth in bouillon was secured in all cases where nitrites were shown to be present.

From this series of tests, it seems that the nitrite formers may lose their oxidizing power when grown on artificial media, and that the bouillon test for purity is uncertain.

Use of $Mg(NH_4)PO_4+6H_2O$ with plate cultures. The suggestion of Stutzer (1901) and Beijerinck (1914) to use $Mg(NH_4)PO_4$ with plate cultures was tested. To the silica plates prepared as before were added 1 cc. each of HCl and Na_2SiO_3 with $Mg(NH_4)PO_4$ solutions properly prepared so that a layer of $Mg(NH_4)PO_4$ silicic acid gel was formed on the surface of the plates. Other tests using the $Mg(NH_4)PO_4$ with agar were made, sterilizing the salt with the agar or adding it sterile to the plates.

In the silica plates it was difficult to see the colonies on account of the particles of salt. No "halo" was noted around the colonies as observed by others. Rarely were positive tests for nitrites obtained. In the agar plates nitrites were found. Colonies on both media gave growth in bouillon.

Soil extract washed agar. One kilogram of soil was sterilized in the autoclave for two hours with 2 liters of water, the liquid pressed through a Chamberland filter and sterilized in the autoclave three times at intervals of twenty-four hours at 15 pounds pressure for four hours. Solution 3 was used with the extract and agar for the medium.

Plates were inoculated with a drop from the 25, 625 and 15,625 dilutions of 1 cc. of a vigorously oxidizing culture of the fifteenth generation. Four kinds of colonies were noted in eight to nine days, picked out with a micropipette (Johnson, 1923), and added to 10 cc. of the agar; this was diluted by transferring a drop to another tube of agar. Plates were made in both cases. Colonies formed in ten days were tested and some showed nitrites present. Two types of organisms were found in these colonies, one a coccus, 1.0 to 1.2 μ and the other a coccus, 0.4 to 0.5 μ in size. The latter seemed to form chains with a sheath. Liquid cultures prepared at the same time as the plates failed to show oxidation after nine weeks. Bouillon tests of the colonies showed purity in only 50 per cent of the cases. Colonies similar to those showing positive nitrite tests were picked out with the micropipette and again plated. Similar colonies were formed, giving positive tests but showing impurity by growth in bouillon.

Further tests were carried out, picking out colonies as before through 9 generations. Similar colonies were formed showing nitrites present but many were impure by the bouillon test. In one test, 50 per cent purity was secured, in two tests, 25 per cent, and in others 10 to 25 per cent. Colonies apparently pure by the test in one generation showed impurity in the next. Apparently the bouillon test is uncertain.

Dilution test. To determine whether the colonies used in these tests contained more than one organism, a single colony was picked out with the micropipette, placed in 10 cc. water with 2 grams pure sand and shaken for three minutes. Dilutions were then made up to 10,000,000. Six experiments were carried out in this way using solutions 1 and 2 and inoculating into bouillon. The 10,000,000 dilution never gave a growth of nitrifiers or showed nitrites present. There was always a growth of actinomyces. The 1,000,000 dilution often showed colonies with nitrites present but actinomyces were invariably present. Dilutions of 10,000 and 100,000 always gave colonies having nitrites present and there were fewer actinomyces. The dilutions below 1000 were nearly always too thickly sown for use. Growth was secured in bouillon up to the 1,000,000 dilution, showing impurity.

The colonies averaged 150 μ in size, ranging from 50 μ in the lower dilutions up to 250 μ in the 100,000 and 1,000,000 dilutions. After the first week growth occurred and the colonies appeared yellowish, circular with an erose edge. Deep colonies were spindle shaped but became circular on coming to the surface. The internal structure was granular and refractive. A thin spreader of film-like growth often surrounded the colonies. It was colorless with a lacerate edge and amorphous internal structure, about 500 μ in size. It did not appear in early dilutions nor with the deep colonies.

Liquid cultures. Solutions 1 and 2 in flasks were inoculated with colonies from soil extract agar plates. In 20 per cent of the inoculations, 1 cc. from dilutions of colonies prepared as described above was used while in other cases the entire colony was used, being picked by a micropipette. In still others 1 cc.

from dilutions of liquid cultures was used. In about 30 per cent of the tests oxidation did not occur; in the majority, it took place in four weeks; in some instances it occurred in two weeks. Where the colony dilutions were used, oxidation was obtained up to the 100,000 dilution but where 1 cc. portions from liquid culture dilutions were used, oxidation took place up to the 1,000,000 dilution. All these cultures as a rule gave growth in bouillon.

Apparently ammonia oxidation takes place in mixed cultures and isolation of the organism is very difficult. It may be stated definitely however, that the ammonia oxidizing organism is a

TABLE 2

Effects of CO_2 on the action of ammonia oxidizer

Dilutions up to and in which NO_2 was present

SERIES NUMBER	SOURCE OF INOCULUM	NORMAL AIR WITHOUT CO_2		NORMAL AIR	AIR DISPLACED BY CO_2	NORMAL AIR WITHOUT OXYGEN	LIQUID MEDIUM
		Soil extract washed agar	Washed agar	Washed agar			
681	Liquid culture	No growth	No growth	1,000	10,000	No growth	1,000,000
682	Liquid culture	No growth	No growth	1,000	10,000	No growth	1,000,000
683	Washed agar plate colony	No growth	No growth	1,000	1,000	No growth	100,000

coccus about 1.2 μ in diameter. The size varies. It is not always regular in form, sometimes being oval. No capsule was observed. In hanging drop it was not found to be motile. It stains well in cold saturated aqueous fuchsin in fifteen seconds.

The effect of CO_2 on growth of organisms. To test the effect of CO_2 and oxygen on the growth of the organism, two liquid cultures and one plate colony were diluted up to 10,000,000 and inoculated into soil extract agar plates with solution 3. One series was kept in desiccators which had an atmosphere of CO_2, formed by the action of H_2SO_4 on Na_2CO_3; another series was kept in a desiccator with all oxygen removed, by pyrogallic acid

and Na_2CO_3, and a third series was kept without CO_2. The results appear in table 2. Without oxygen and without CO_2 there was no oxidation; but with excess of CO_2, oxidation occurred in greater dilutions than with normal air. Evidently the soil extract did not serve as a source of carbon, where CO_2 was not available. Neither did the basic carbonate of solution 3 serve to supply carbon. It is free CO_2 which the organism uses. This is in conformity with the conclusions of Winogradsky and Omeliansky (1899), Godlewski (1892, 1895, 1896) and Bonazzi (1921).

Isolation of the nitrate formers

The following nutrient solution was used (Gibbs, 1919):

Solution 11

$NaNO_2$	1.0 gram
Na_2CO_3	1.0 gram
K_2HPO_4	0.5 gram
NaCl	0.5 gram
MgSO	0.3 gram
$Fe_2(SO_4)_3$	Trace
Water (conductivity)	1000 cc.

Flasks of this solution were inoculated with 1 gram of soil and the presence of nitrate tested by diphenylamine. In ten days all the nitrite was oxidized in practically all cases. Five cubic centimeters of the NO_2 solution were added to each culture when the nitrite had disappeared and this was repeated twenty-five times. The average time to oxidize the 5 cc. of nitrite was four to five days. Tests in bouillon showed growth in all cases.

Subcultures obtained by inoculating 1 cc. of the first cultures into fresh solutions, were carried through seventeen generations. All gave growth in bouillon. The average time taken for each generation to oxidize 25 cc. of solution varied from nine to sixteen days.

Dilution tests. Fifteen cultures carried through eight subcultures were diluted 25, 625, 15,625, 390,625, and 9,765,625 times and 1 cc. inoculated into Nährstoff-Heyden solution, bouillon and no. 11. Some of the cultures prepared from the higher dilutions from which growth did not occur in bouillon or

Nährstoff-Heyden solution were inoculated into fresh solution 11. The nitrite was oxidized but growth occurred in bouillon. The mother culture did not grow in bouillon while the subculture did. The bouillon test does not seem to be constant.

Washed agar and silicic acid gel. On these solid media light yellow microscopic colonies about 500 μ in size appeared in three weeks. The surface colonies had somewhat indefinite edges and a heart-shaped granular center. Cultures were made from these colonies, in no. 11 and sometimes nitrates appeared in seven days. Occasionally all the nitrite was oxidized in sixteen days. Subcultures were prepared and carried through six or eight generations. All gave growth in bouillon. Oxidation occurred in all cases and was completed in varying periods, ranging from two to three weeks. Organisms from vigorously oxidizing cultures were found to be short rods, about 0.8 μ long and 0.4 μ broad. No motility was observed.

Sand cultures

Bazarewski (1906) Coleman (1908), and Wimmer (1904) used sand in studying the nitrifiers. In this work washed sand was sterilized in petri dishes with solutions 1 and 2 and inoculated with cultures of both the nitrite and nitrate formers. Nitrites were found in ten days and in fourteen days both nitrites and nitrates were present. In eighteen days no nitrites were present in 75 per cent of the plates. A similar experiment using 6 inches of sand in beakers and in flasks gave very much the same results.

Action of nitrifiers in presence of growing plants

The favorable results obtained in regard to rapid oxidation of ammonia nitrogen to nitric nitrogen led to the following experiment.

A battery jar 14.5 cm. in diameter and 18 cm. high (fig. 1) was fixed with a specially made cover *PP* of burnt clay, provided with a groove *AA* to fit the battery jar edge *JJ*. In the center of the cover there was a hole *B* 1.5 cm. in diameter and around this hole a groove *CC* 0.5 cm. deep and 5 cm. in diameter.

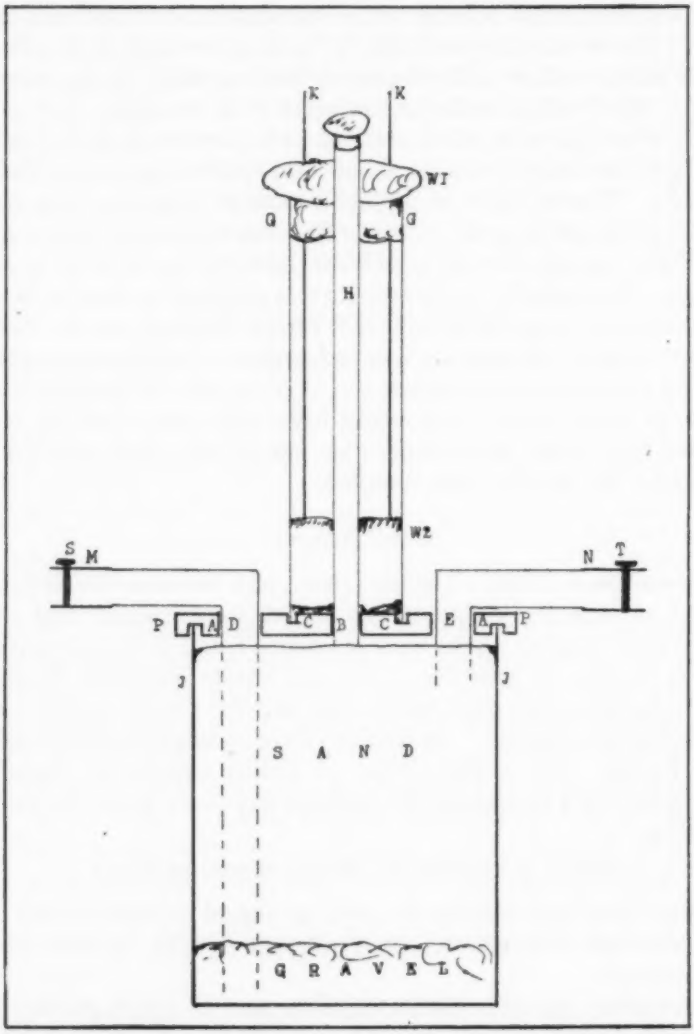

FIG. 1. DIAGRAM OF APPARATUS FOR GROWING PLANTS UNDER STERILE CONDITIONS

There were two other holes *D* and *E* 2 cm. in diameter placed near the edge of the cover. In the groove *A* between the cover and the battery jar cotton was placed. A tube bent at right angles passed through *D* and reached the bottom of the jar. A similar tube passed through *E* but extended only 3 cm. into the jar. Cotton was placed around these tubes. The outer ends of the tubes were provided with rubber tubing *M* and *N* and screw clamps *S* and *T*. Through *B* 500 grams of clean, washed dry gravel was poured into the jar and evenly distributed on the bottom. On top of it 2750 grams of sand cleaned in dilute HCl, washed in distilled water and dried, was poured in. A glass cylinder *GG*, 5 cm. in diameter and 20 cm. in length, fitted into groove *CC*. A glass tube *H* 1.5 cm. in diameter and 25 cm. in length passed through *B* and rested on the sand. The top of this tube carried a cotton plug. Between the two cylinders there were loose cotton packings W_1 and W_2 at top and bottom and three glass rods *KK* about 0.4 cm. in diameter and 20 cm. in length.

The entire apparatus was sterilized in the autoclave three times on alternate days at 15 pounds pressure for three hours.

The solutions that produced the maximum growth of wheat according to Skinner and Reid (1921) contained phosphate nitrate and potash in the ratio of 20:40:40. In this work $CaH_4(PO_4)_2$, $NaNO_3$ and K_2SO_4 solutions with a concentration of 160 parts per million of P_2O_5, NH_3 and K_2O were made. The solutions were sterilized separately and then mixed in equal parts. For every 100 cc. of the solution, 1 cc. of the liquid cultures of the nitrite and nitrate formers were added. Enough solution was added through tube *D* to bring the moisture content of the sand to the optimum.

Wheat seeds were sterilized by washing in 1:500 $HgCl_2$, 50 per cent alcohol and sterile water. The cotton plug was removed from tube *H* and two seeds were dropped in. When the seedlings showed up above the cotton packing W_2, the tube *H* was slowly pulled up and the cotton was packed around the seedling by the glass rods *KK*. The jars were then removed to the greenhouse. Sterile water was added through *D* as evaporation took place.

The seedlings grew to maturity and formed heads as under normal conditions. At the end of the growing period the sand was tested for nitrates, nitrites and ammonia. Large amounts of nitrates and traces of ammonia were present. Evidently the nitrifying organisms can function in the presence of growing plants.

Contaminating forms

Bacterial forms. All investigators have found that contaminating forms persist in cultures of both the nitrite and the nitrate formers. The organisms that contaminate the nitrate former are more or less fully described, but there is no detailed description of the organisms that contaminate nitrite formers. In this work the organisms contaminating the nitrite formers are described.

In the earlier part of the work two kinds of growth were noticed in bouillon when it was inoculated with colonies of nitrite formers. There was a slight turbidity accompanied by the formation of a slimy film at or near the surface. The other growth was much heavier consisting of coarse orange granules and a very thin bluish film climbed the sides of the tube. The former is a coccus and the latter a short rod.

Coccus

I. Morphology

Form: The organism was definitely spherical in shape.

Size: Forty-eight hour agar cultures of the organism showed individual cells most of which were from 0.4 to 0.5 μ in diameter. Often these were found in pairs. The size of the organism varied little with age or change in media. On potato the organisms appeared in chains of 3 and 4, often bent and covered with a sheath-like membrane.

Motility: No motility was found in hanging drop.

Staining reactions: Easily stained by ordinary stains. Saturated aqueous fuchsin gave good stains in fifteen seconds. The Gram stain was positive.

Spore formation: Both young and old cultures failed to resist 80°C. for ten minutes. No evidence of spores noted.

II. Cultural characteristics

Agar streak: Scanty growth. Very small colonies grew along the streak and did not run together. They were convex, refractive, light yellow or colorless. No odor was observed. The growth was brittle.

Agar plate colonies: In ten days about 300 μ in size. Oval and heart shaped with rough and raised surface. Edge entire and internal structure granular.

Nutrient broth: Two or three days slight turbidity with a thin film on the surface. Later the film grew heavier and on shaking fell to the bottom of the tube. Mucilagenous and appeared fleecy, yellowish in color.

Nutrient agar plate colonies: Growth slow, form circular or oval, surface rough, elevation raised, edge erose, internal structure coarsely granular, size twelve days 140 μ.

Gelatin stab: Thin bluish growth on surface next to walls of tube in twelve days; no growth along the line of puncture. In four to six weeks cultures, heavier and yellowish granular. No liquefaction.

Gelatin plate colonies: In three days colonies 35 to 100 μ; circular and raised with an entire edge; no liquefaction; internal structure first amorphous later finely granular, refractive and light yellow in color.

Glucose agar slant: Growth moderate; form echinulate; elevation flat; luster glistening; topography contoured; chromogenesis grayish or cream colored; odor absent; consistency viscid; medium not changed.

III. Physiology

Chromogenesis: Light yellow on potato and in nutrient broth; bluish first, then light yellow on gelatin, none on agar. Production of indol in bouillon in six days.

Relation to oxygen: Obligate aerobe.

Fermentation: Grew well in lactose, sucrose and glucose; slight acid was observed in last two media. Gas was not produced.

Milk: In two weeks milk curdled and peptonized.

Nitrate reduction: Not noticed though slight fleecy growth at bottom of tube.

(Index number[3] 3331—51235—2332—1122—00—313—11—121.)

[3] According to the Descriptive Chart adopted by the Society of American Bacteriologists, December 30, 1920.

Short rod B

I. Morphology

Form and size: Forty-eight to seventy-two hour cultures of the organisms showed individual cells, most of which were short rods from 0.6 to 0.7 μ in length, and 0.5 to 0.6 μ in width.

Arrangement: Many isolated cells, pairs frequent and chains of 3 or 4 also common.

Motility: Not observed.

Staining reactions: Stained readily with ordinary stains.

Spore formation: Not observed, and both young and old cultures failed to resist 80°C. for ten minutes.

II. Cultural characteristics

Agar stroke: Growth scanty and microscopic; filiform and beaded; elevation, flat; luster, dull; topography, slightly bullate; refractive; consistency, brittle; dull cream color; no coloration of medium; rate of growth slow—only slight growth in three days.

Agar plate colonies: Growth slow; punctiform; surface rough; elevation convex; edge erose; internal structure coarsely granular.

Gelatin stab: Orange granular growth on surface and none along the line of puncture; no liquefaction; medium not colored; rate of growth slow—no growth in three days; in two weeks growth increased and began to climb the walls of the tube.

Gelatin plate colonies: Observed in three days; form irregular; size 200 to 300 μ, some aggregates of colonies 1 to 2 mm.; elevation convex; edge erose; liquefaction none; internal structure coarsely granular.

Nutrient broth: On the surface orange-yellow granules and a thin pale film that climbed up the sides of the tube. No clouding in the liquid unless tube was shaken. No odor or sediment, the granules fell to the bottom but none grew at the bottom. Growth observed in forty-eight to seventy-two hours.

Glucose agar slant: Growth abundant; form effuse; elevation raised; dull appearance; topography bullate; opaque; orange yellow; odor none; consistency brittle.

III. Physiology

Chromogenesis: Orange yellow on nutrient broth, nutrient gelatine and potato.

Production of indol: Observed in six days.

Relation to oxygen: Obligate aerobe.

Diastatic action: Slight.

Fermentation: Grew well in lactose, sucrose and glucose, but without producing gas or acid. Growth at the surface while liquid clear.

Milk: Orange granular growth on surface; no action on medium. (Index number 5331—51236—1333—2112—00—322—13—322.)

Actinomyces were found in all the generations of cultures of nitrite formers. In five generations of dilution cultures ranging up to 10,000,000, it was observed that in dilutions of 10,000 and above the number of actinomyces colonies increased. In the lower dilutions the growth of actinomyces seemed to be inhibited.

Three of the most typical actinomyces colonies that were observed in all the cultures above studied were picked and their cultural and morphological characteristics determined. The method of study adopted is virtually the same as that of Waksman (1919).

Actinomyces 13

I. Morphology

1. Spirals, none; short, straight, branching mycelium 0.6 to 0.7 μ by 5–7 μ.

2. Conidia. Egg-albumen agar; oval 0.4 μ by 0.6 μ.

II. Cultural characteristics

1. *Synthetic agar:* Growth; moderate spreading, colonies less than 1 mm. Aerial mycelium; in clumps, white in color. No soluble pigment.

2. *Calcium acetate glycerine agar:* Growth, scanty; aerial mycelium, white; no soluble pigment.

3. *Nutrient agar:* Growth moderate in media—colonies like specks. Aerial mycelium, cream white specks. No soluble pigment.

4. *Loeffler's blood serum:* Growth, scanty; aerial mycelium, white specks; soluble pigment, brown; liquefaction, rapid.

5. *Starch agar:* Growth, small specks on surface; no growth below surface of medium. Aerial mycelium, short broken white clumps. No soluble pigment. Diastatic action, very good.

6. *Potato:* No growth.

7. *Gelatin stab:* Growth, numerous cream white colonies in a ring at the surface, some at bottom. Aerial mycelium, white. Liquefaction, rapid.

8. *Litmus milk:* Growth, small colonies on sides of tube; little sediment. No aerial mycelium. No peptonization.

9. *Plain milk:* Growth, small colonies in liquid at surface and bottom. No aerial mycelium. No coagulation. No peptonization.

10. *Lactose broth:* Growth, slight or none in liquid.

11. *Glucose broth:* Growth, abundant at top; liquid clear. Aerial mycelium, white. Soluble pigment, pink brown.

12. *Glucose slant:* Growth, effuse surface growth, wrinkled and leathery. Aerial mycelium, brown.

13. *Glucose agar plate:* Small speck-like growth. Aerial mycelium, white. Soluble pigment, yellow.

14. *Glycerine asparaginate agar:* Growth spreader in medium. Aerial mycelium, white specks. No soluble pigment.

15. *Egg albumen agar:* Growth, compact center colonies with fern-like edges. Aerial mycelium, white specks. No soluble pigment.

16. *Glycerine nitrate agar:* Growth, light yellow, scanty. Aerial mycelium, cream white. No soluble pigment.

17. *Starch gelatin stab (pH 6.4):* Growth, speck-like colonies at top; heavy sediment at bottom. No aerial mycelium. Soluble pigment, light yellow. Change of reaction, alkaline.

18. *Czapek's modified or synthetic solution:* Growth, numerous speck-like colonies at top, in the liquid, on the wall and at the bottom. Aerial mycelium, white. Soluble pigment, yellow.

19. *Bouillon:* Growth, at bottom—numerous small speck-like colonies some on surface wall—liquid clear. No aerial mycelium. Soluble pigment, yellowish-orange.

20. *Nitrate broth:* Growth, small colonies all through liquid and on the wall above the surface. Aerial mycelium, small white specks. Soluble pigment, yellow. Nitrate production heavy.

Actinomyces 11

I. Morphology

1. *Spirals:* Usually none on media studied. Mycelium consists of straight branching hyphae. Occasionally an open spiral is noted.

2. *Conidia:* Synthetic agar. Oval 1.3 by 0.7 μ. Also found on glucose agar, glycerine apparaginate, and glycerine nitrate agar.

II. Cultural characteristics

1. *Synthetic agar:* Growth: Compact colonies with few mycelia sticking out like bristles. Size of colonies less than 1 mm. Aerial mycelium: White and straight. No soluble pigment.

2. *Calcium acetate glycerine agar:* Growth: Spreads below the surface speck-like colonies. Aerial mycelium: White, long and scanty. Soluble pigment: None, sometimes rose.

3. *Nutrient agar:* Growth: Colonies 2 to 3 mm. in size. Abundant growth below surface. Aerial mycelium: White. Soluble pigment: Dark brown, sometimes none.

4. *Loeffler's blood serum agar:* Growth: Effuse, yellowish colored glossy surface. Aerial mycelium: White or cream colored. Soluble pigment: Slight, yellow. Liquefaction: Slow.

5. *Starch agar plate:* Growth: Abundant spreader in medium. Aerial mycelium: White. Enzymatic action: Good.

6. *Potato:* Growth: None at room temperature, but at 35°C. echinulate growth in two weeks. Aerial mycelium: White and gray. Color of potato: No change.

7. *Gelatin stab:* Growth: At top; after liquefaction sediment is noted at bottom. Aerial mycelium: White. Soluble pigment: Yellowish brown. Liquefaction: Rapid.

8. *Litmus milk:* Growth: On surface—small amount of sediment. Aerial mycelium: White. Coagulation: Slow. Peptonization: Slow; complete in two weeks. Change of reaction: Alkaline. Soluble pigment: Lavender.

9. *Plain milk:* Growth: At surface—small amount of sediment. Aerial mycelium: White. Coagulation: Slow. Peptonization: Complete in nine days. Change of reaction: Alkaline. Soluble pigment: Brown.

10. *Lactose broth:* Growth: Abundant on surface. Aerial mycelium: White. Soluble pigment: None, sometimes brown.

11. *Glucose broth:* Growth: Abundant on surface. Aerial mycelium: White. Soluble pigment: Dark brown.

12. *Glucose slant:* Growth: Echinulate. Aerial mycelium: Cream white. No soluble pigment.

13. *Glucose agar plate:* Growth: Abundant spreader in medium. Aerial mycelium: White (bristling). Soluble pigment: yellow.

14. *Glycerine asparaginate agar:* Growth: Inside medium. Aerial mycelium: White specks. No soluble pigment.

15. *Egg albumen agar:* Growth: In medium. Aerial mycelium: White. No soluble pigment.

16. *Glycerine nitrate agar:* Growth: Heavy—colonies run together—spread in medium. Aerial mycelium: White. Soluble pigment: Purple (iodine) sometimes none.

17. *Starch gelatine stab* (pH 6.4): Growth: At the bottom and on surface of liquid. No aerial mycelium. No soluble pigment. Change in reaction: Alkaline.

18. *Czapek's modified or synthetic solution:* Growth: Cream colored surface ring and small amount of sediment at bottom. Aerial mycelium: Cream white. Soluble pigment: Light red.

19. *Bouillon:* Growth: Small surface ring: (Liquid clear) Aerial mycelium: White. Soluble pigment: Reddish brown.

20. *Nitrate broth:* Growth: Speck-like colonies on surface; some in medium. Aerial mycelium: White or cream white. Soluble pigment: Light yellow, sometimes rose. No NO_2 production.

Actinomyces 12

I. Morphology

1. *Spirals:* None on media studied; aerial mycelium, only long straight branching hyphae.

2. *Conidia:* Oval 0.7 to 0.8 μ by 1.0 to 1.2 μ.

II. Cultural characteristics

1. *Synthetic agar:* Growth: Abundant spreading growth; penetrating into medium. Aerial mycelium: White and gray. No soluble pigment.

2. *Calcium acetate glycerine agar:* Growth: Scant and slow. Aerial mycelium: White. No soluble pigment.

3. *Nutrient agar:* Growth: Compact colony, slight penetration into medium, membranous on surface. Aerial mycelium: White. Soluble pigment: Whitish brown.

4. *Loeffler's blood serum:* Growth: Glossy round yellowish colonies with depression in center. No aerial mycelium. No liquefaction.

5. *Starch agar:* Growth: Spreading in the medium. Aerial mycelium: Cream white. No soluble pigment.

6. *Potato:* Growth: None at room temperature, but at 35°C. echinulate growth. Aerial mycelium: White, heavy and all over growth. Color of potato: Unchanged.

7. *Gelatin:* Growth: Formation of heavy surface ring and a heavy sediment. No aerial mycelium. Soluble pigment: Orange yellow. Liquefaction: Rapid.

8. *Litmus milk:* Growth: Surface ring of small colonies. No aerial mycelium. Coagulation: Slow. Petponization: Slow, first observed after four weeks. No change in reaction.

9. *Plain milk:* Growth: Slow and scant—not noticed for four weeks. No aerial mycelium. Coagulation: After six weeks. No peptonization.

10. *Lactose broth:* Growth: Heavy mat of colonies on surface. Liquid clear; no sediment. Aerial mycelium: White and all over the growth. No soluble pigment.

11. *Glucose broth:* Growth: Heavy mat of colonies on surface, liquid clear; no sediment. Aerial mycelium: White all over the growth. No soluble pigment.

12. *Glucose slant:* Growth: Echinulate. Aerial mycelium: White and gray—raised. No soluble pigment.

13. *Glucose agar plate:* Growth: Heavy—spreading in medium and on the surface. Aerial mycelium: Chalk white. No soluble pigment.

14. *Glycerine asparaginate agar:* Growth; radiating. Aerial mycelium: White. No soluble pigment.

15. *Egg albumen agar:* Growth: Heavy; spreading in medium. Aerial mycelium: White and gray. No soluble pigment.

16. *Glycerine nitrate agar:* Growth: Compact. Aerial mycelium: White, straight, projecting from colonies like bristles. No soluble pigment.

17. *Starch gelatine stab (pH 6.4):* Growth: None at surface, but 2 or 3 colonies about 1 cm. in diameter at bottom. No aerial mycelium. No liquefaction. No change of reaction.

18. *Czapek's modified or synthetic solution:* Growth: Heavy mat of small colonies on surface; colonies all over the surface of tube in liquid and a mat of colonies at bottom. Aerial mycelium: White and gray. No soluble pigment.

19. *Bouillon:* Growth: One big colony covering the whole surface of liquid which is clear. Aerial mycelium: White. Soluble pigment: Orange brown.

20. *Nitrate broth:* Growth: Colonies floating in liquid and some sticking to bottom of tube; liquid clear. No aerial mycelium. Soluble pigment: Light green. NO_2 production: Heavy.

SUMMARY

In spite of the extensive work done in the isolation of nitrifying organisms, the methods are quite unsatisfactory. In this work the isolation of nitrite formers was attempted, first, by carrying on a long series of subcultures which failed to give bouillon sterile cultures. The contaminating forms were present with the nitrite former in the loop inoculum. The subculture method was modified by diluting the cultures from 1:10,000,000, and using the dilutions as inoculum. This method also proved inadequate. Next solid media, washed agar, soil extract agar, and silicic acid gel were used. Different combinations of nutrient salts were tried with these media. Soil extract agar gave the most rapid growth of the organisms and the best oxidation of ammonia. But the colonies on this medium, like those on others, contained nitrite formers that gave sporadic growth in bouillon. This was proved by picking out a single colony by a micropipette, breaking the organisms loose in sand water blanks and diluting from 1:10,000,000 times and inoculating on soil extract agar plates. The growth in the plates contained besides colonies of the type used originally, actinomyces and two bacterial forms. All of these contaminators are described.

The fact that actinomyces always appear in the higher dilutions and are always closely associated with nitrite formation appears to be quite significant. No difficulty was encountered in securing pure cultures of these organisms, but they never showed any power of oxidation. They would grow in bouillon at the surface and sometimes at the bottom, but never produced any turbidity.

The nitrite formers were grown in atmospheres with and without CO_2 and oxygen. Oxidation of ammonia was not noted where CO_2 and oxygen were absent.

Isolation of the nitrate formers was carried out on the same lines as those adopted in the isolation of nitrite formers, making preliminary enrichment cultures, then subcultures and dilution cultures and finally inoculating these into two solid media, silicic acid gel and washed agar.

Nitrite formers and nitrate formers in liquid cultures were inoculated into sterile sand supplied with nutrient salts. Wheat seeds were planted. The ammonia was oxidized to nitrate and the wheat plant made normal growth.

CONCLUSIONS

1. The bouillon test for purity of nitrite formers and nitrate formers is not conclusive due possibly to the fact that the organisms develop tolerance for bouillon.
2. The nitrite formers may rapidly lose their power of oxidation of ammonia when grown in pure cultures.
3. Free CO_2 is essential for oxidation of ammonia by nitrite formers.
4. CO_2 promotes ammonia oxidation better than normal air.
5. Nitrite formers grow most rapidly in soil extract washed agar. The carbonaceous material in soil extract does not act as a source of carbon to nitrite formers, when free CO_2 is not available.
6. The nitrifying organisms oxidize ammonia to nitrite and nitrite to nitrate in the presence of growing plants.

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THE NUMBER OF GENERATIONS NECESSARY FOR THE DEVELOPMENT OF THE POWER OF AEROBIC GROWTH BY BACTERIUM ABORTUS (BANG)

AMANDA HARMS

Laboratory, Department of Animal Husbandry, Kentucky Agricultural Experiment Station

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It is a well known fact that the Bang bacterium when first isolated does not grow readily in the presence of air. The employment of *B. subtilis* as an agent in the production of partial anaerobiosis was found to be necessary in growing this organism until several generations have passed. A glass jar may be used from which the air can be partly exhausted with a suction pump but we prefer in this laboratory the use of *B. subtilis* which is the cultural method of Nowak of Austria (1908). This method consists in lowering the pressure of oxygen to that necessary for the growth of the abortion bacterium by means of the *Bacillus subtilis*, allowing 1 sq. cm. of surface culture of the *B. subtilis* to each 15 cc. capacity of the sealed jar in which it is grown.

The technique usually employed is to streak the contaminating material over two or three Petri dishes in succession, as is ordinarily done in making streak dilutions. The plates are then incubated at 37°C. for twenty-four hours in the air to allow contaminating organisms to develop. The colonies are then marked with a wax pencil or India ink and the plates transferred to the Novy jar and incubated for three days, as indicated above. At the end of this time the transparent colonies of *Bact. abortus* (Bang), if present, may appear on the clear spaces.

In our studies to determine the number of generations it takes to grow this organism in air, we incubated the plates in air for three days to give the *Bact. abortus* (Bang) ample time to grow.

If no colonies of the Bang bacterium were visible at the end of this time, the plates were incubated in the Novy jar for three days. After the *Bact. abortus* (Bang) becomes accustomed to growing in the air, it requires but two days to grow abundantly and even after twenty-four hours the growth is quite noticeable.

The H-ion concentration is an important factor in growing the *Bacterium abortus* (Bang). The limiting H-ion concentration in which growth may be obtained lies between pH 6.0 and 7.6 (Huddleson, 1920). We have found it to be very satisfactory between pH 6.6 and 7.0 and have used plain 2 per cent beef agar with an H-ion concentration of pH 7.0 in our work.

Fabyan (1912) states that sometimes this organism will grow in the air when first isolated. He says: "It is probable that strains will be found to vary more or less in their relation to oxygen and to the kinds of culture media usually employed. For instance, culture II, a and b, we were able to isolate without *B. subtilis* directly from guinea-pigs inoculated with the original material."

Surface (1913) in 1911, isolated cultures of *Bact. abortus* (Bang) from an afterbirth and fetus of a cow that was injected subcutaneously with 20 cc. of a dilute mixture of three Danish strains of *Bact abortus* (Bang) which had been grown aerobically in the laboratory for more than two years. Typical abortion was produced fifty-two days after the injection. Cultures from the aborted material grew readily in air. Growth appeared on plain agar on the second day after incubation.

In February, 1916, Good and Smith isolated the *Bact. abortus* (Bang) from the fetus and afterbirth of an aborting sow. This organism grew in the air after the first generation. When streaked on agar slants and incubated for forty-eight hours, some little growth could be detected even with the naked eye, and at the end of seventy-two hours quite a luxuriant growth of the organism was obtained. None of the material from this aborting sow was incubated aerobically for any length of time; therefore Good and Smith could not state whether the organism isolated would have grown directly from the tissues in the first generation, but we are sure that it would have because the

organism was recovered in inoculation experiments and grew in air in the first generation, and since that time we have isolated *Bact. abortus* (Bang) aerobically from the fetus of an aborting sow.

Stafseth (1920) was unable to isolate *Bact. abortus* (Bang) under aerobic conditions. Fitch (1922) states that after two or

TABLE 1

STRAINS OF BACT. ABORTUS BANG	DATE ISOLATED	AEROBIC GROWTH												
		First generation	Second generation	Third generation	Fourth generation	Fifth generation	Sixth generation	Seventh generation	Eighth generation	Ninth generation	Tenth generation	Eleventh generation	Twelfth generation	All other generations
	1920													
D1	Aug. 9	0	0	0	0	0	0	+	0	0	+	0	0	++
D2	Sep. 14	0	0	0	0	+	0	0	0	0	+	0	++	++
N	Dec. 18	0	0	0	0	0	0	0	0	0	0	0	0	++
B	Oct. 9	0	0	0	0	++	++	++	++	++	++	++	++	++
	1921													
B.C. I	Aug. 12	0	0	0	0	0	0	++	++	++	++	++	++	++
	1922													
B.C. II	Mar. 13	0	++	++	++	++	++	++	++	++	++	++	++	++
B.C. III	Apr. 12	++	++	++	++	++	++	++	++	++	++	++	++	++
A	May 24	0	0	+	0	0	0	0	++	++	++	++	++	++
D3	Nov. 24	0	0	0	++	++	++	++	++	++	++	++	++	++
D4	Dec. 18	0	0	++	++	++	++	++	++	++	++	++	++	++
D5	Dec. 26	0	++	++	++	++	++	++	++	++	++	++	++	++
	1923													
D6	Apr. 10	++	++	++	++	++	++	++	++	++	++	++	++	++
D7	Oct. 4	0	0	+	0	+	Not cultured							

0 = no growth, + = scanty growth, ++ = abundant growth.

three transfers *Bact. abortus* grows quite readily even under ordinary aerobic conditions. Alice C. Evans (1918) states that the first growth of a strain transferred from pathologic material to artificial media is often difficult to obtain. Growth is favored by incubation in a closed jar with *B. subtilis*. Glycerol agar or serum agar serves well for such a strain, but after it becomes

accustomed to artificial conditions growth is abundant on all the ordinary media.

We have made a study of 13 different strains of *Bacterium abortus* (Bang) and have found them to vary greatly in the number of generations it took to grow in the air. These cultures have been isolated from fetuses and after births that were sent to the laboratory from aborting cows. The results are shown in table 1.

We have had two strains of *Bact. abortus* (Bang), B.C. III and D6, that grew in the air in the first generation, and two strains, B.C. II and D5, that grew in the air in the second generation. In the case of three strains, B.C. II, B.C. III, and D5, the live organism of swine origin isolated by Good and Smith in 1916 had been injected, and in the case of strain, D6, the live organism of bovine origin had been injected. These cultures had been grown aerobically in the laboratory for many generations. This leads us to believe that the injections of the live organism were the cause of the abortions. At any rate, the strains of *Bact. abortus* isolated from these animals grew readily in the air in the first and second generations.

We cannot account for the peculiar actions of *Bact. abortus* (Bang) D1, D2, and A. It may be that there was a slight difference in the H-ion concentration, or possibly in the moisture content of the agar used, that caused D1 to give a scanty growth in the seventh generation, no growth in the eighth and ninth, very scanty growth in the tenth generation, no growth in the eleventh and twelfth generations, thus making it necessary to cultivate it in the Novy jar by the Nowak method, until the thirteenth generation was grown. After that time it grew readily in air.

Bact. abortus (Bang) D2 and A acted much the same, giving a slight growth in air in certain generations, no growth in others, and finally grew readily in the air, D2 after the twelfth generation and A after the sixth generation.

It is evident that the acquired character of this organism to grow aerobically is transmitted to its descendants.

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STUDIES ON PASTEURIZATION

XI. THE "MAJORITY" AND "ABSOLUTE" THERMAL DEATH-POINTS OF BACTERIA IN RELATION TO PASTEURIZATION

S. HENRY AYERS AND W. T. JOHNSON, JR.

From the Research Laboratories of the Dairy Division, United States Department of Agriculture

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In earlier papers (Ayers and Johnson, 1910; 1913; 1914; 1915) the importance of the "majority" thermal death-point of bacteria has been discussed in relation to the development of bacteria in pasteurized milk. There now appears another important relation of this subject to the process of pasteurization which there has been a tendency to overlook.

The growing inclination is to test the efficiency of the pasteurizing process by elaborate large scale experiments in which the effectiveness of the process is measured by the destruction of pathogenic organisms artificially inoculated in large numbers into the milk. In such experiments only relatively small amounts of the total volume of milk pasteurized can be subjected to laboratory tests. The question, therefore, arises as to how the "majority" and "absolute" thermal death-points of bacteria are related to the interpretation of the results of such experiments.

When bacteria are heated to temperatures above their growing range, a degree of heat is reached where a few cells are killed. With further increase in temperature the destruction continues and the temperature at which the majority of the cells are killed has been termed, by Gage and Stoughton (1906), the "majority" thermal death-point. This thermal death-point must not be confused with the "absolute" thermal death-point which is the degree of heat required to kill all living cells. Both the "major-

ity" and "absolute" thermal death-points of bacteria will vary in different media of varying composition and hydrogen ion concentration and in this paper they will be considered only in relation to milk.

The following series of experiments, which is one of several giving similar results, well illustrates the important significance of the difference between "majority" and "absolute" thermal death-point of a culture of *B. aerogenes*. Flasks each containing 100 cc. of skimmed and whole milk were prepared and heavily inoculated with 2 cc. from an eighteen hour old culture of *B. aerogenes* grown in a 0.5 per cent pepton solution. Two flasks, one containing skimmed milk and the other whole milk, were heated to 135°F. and held for thirty minutes in a water bath. The contents of each flask were carefully agitated in such a way as to insure an even temperature and at the same time keep the milk in the flask far below the heating surface, in other words, below the surface of the water in the bath. Particular care was taken therefore to be sure that none of the milk escaped heating.

Counts made by direct plating of the contents of each flask showed the following results:

	BACTERIA PER CUBIC CENTIMETER	
	Before heating	After heating to 135°F. for thirty minutes
Skimmed milk	4,000,000	237
Whole milk	4,300,000	53

It will be seen from the figures that the majority of the cells were destroyed at 135°F. but that a sufficient number survived so that they were present in 1 cc. of milk and could readily be detected by ordinary plating methods. The actual "majority" thermal death-point of the culture was evidently lower than 135°F. and would have been at the point where a little over one-half of the cells were killed.

The experiment was repeated using a temperature of 140°F. for a period of thirty minutes with the following results:

	BACTERIA PER CUBIC CENTIMETER	
	Before heating	After heating to 140°F. for thirty minutes
Skimmed milk	4,500,000	None in 1 cc.
Whole milk	4,300,000	None in 1 cc.

It might appear from these results that it could be assumed that the cells were all destroyed because they could not be detected by plating 1 cc. However, such an assumption, on purely theoretical grounds, would be unsafe to make because 100 cc. of milk constituted the amount of infected milk being studied and only 1 cc. was examined for the presence of living organisms. Arguing again, on theoretical grounds, from the fact that 1 cc. out of a total volume of 100 cc. contained no living organisms it could not be said with certainty that there remained no living cells in the remaining 99 cc.

Further experiments with the milk heated to 140°F. for thirty minutes showed that the cells were not all destroyed even though they could not be detected by examining 1 cc. or 1 per cent of the total volume of infected milk. In order to determine this point flasks containing 50 cc. of sterile milk were inoculated with 2, 3, and 4 cc. of the heated infected milk. Upon incubation of these flasks it was found that in each flask *B. aerogenes* developed, showing therefore its presence in as low an amount as 2 cc. of the milk. It is evident that by heating the infected milk to 140°F. for thirty minutes a sufficient number of cells survived to be detected by examination of 2 cc. of milk even though they could not be found by plating 1 cc.

Since 140°F. was not sufficiently high to kill all the cells, a flask containing 100 cc. of skimmed milk was inoculated and heated to 145°F. for thirty minutes. Plating results were as follows:

	BACTERIA PER CUBIC CENTIMETER	
	Before heating	After heating to 145°F. for thirty minutes
Skimmed milk	4,600,000	None in 1 cc.

As was expected from the previous experiment no living organisms were found by plating 1 cc. so 2, 3, 4, and 5 cc. of the heated infected milk were inoculated into tubes each containing about 5 cc. of sterile litmus milk. Since upon incubation these tubes all remained sterile it was evident that there were no living cells in 14 cc. of the heated milk. It should be remembered that this means that 14 per cent together with 1 per cent used in direct plating or a total of 15 per cent volume of the milk, when examined, showed no evidence of living organisms which survived the heating.

After removing the 15 cc. from the total volume of 100 cc. of heated milk there remained in the flask 85 cc. This was allowed to incubate and it showed a development of the *B. aerogenes* culture. It is evident, therefore, that the cells were not all destroyed by heating to 145°F. for thirty minutes, although they could not be detected by plating 1 cc. or by inoculation of an additional amount of 14 cc. into sterile milk.

The "absolute" thermal death-point of the culture of *B. aerogenes* studied was found to be a little above 150°F. when the milk was heated for a period of thirty minutes, the "absolute" thermal death-point being the temperature at which all living cells of the culture are killed.

DISCUSSION OF RESULTS

The "majority" thermal death-point of the *B. aerogenes* culture studied was below 135°F. and the "absolute" thermal death-point a little above 150°F. when the temperature was maintained for a period of thirty minutes. When 100 cc. of infected milk was heated at 135°F. surviving cells could be detected by examining 1 cc. or in other words by examination of 1 per cent of the total volume of milk studied. When heated to 140°F. it was necessary to examine 2 per cent of the milk to find surviving cells. At 145°F. for thirty minutes no living organisms could be detected when 1 per cent (1 cc.) was examined nor when in addition 14 per cent of the total volume of milk was examined, but there were surviving cells in the remaining 85 per cent of the total volume.

How can these results be interpreted as they are related to large scale efficiency tests of the pasteurizing process? Suppose a test is being made in which instead of 100 cc. of milk infected with *B. aerogenes* there had been 1000 pounds. Calculating the results on this basis when heating to 140°F. 10 pounds of milk, approximately 4500 cc. or 1 per cent of the total volume could have been examined without detecting the survival of living cells, although they could have been found by the examination of 2 per cent of the total volume this being 20 pounds or about 9000 cc. In a test therefore with 1000 pounds of milk the examination of 4500 cc. of heated milk would, on the basis of the results presented, have shown no living cells of the test culture and a temperature of 140°F. for thirty minutes would therefore be considered effective for destroying all cells. The seriousness of the interpretation of results in this manner is at once evident when it is remembered that similar differences between the "majority" and "absolute" thermal death-points probably exist among pathogenic bacteria.

In a similar manner the results obtained by heating the *B. aerogenes* culture to 145°F. for thirty minutes may be calculated on the basis of a larger experiment using 1000 pounds of milk. It will be remembered that no living cells were found when 15 per cent of the total volume of heated infected milk was examined but were present in the remaining 85 per cent. Calculating on the basis of 1000 pounds of milk, it is evident that bacteriological studies of as much as 15 per cent (150 pounds) or about 67,500 cc. would not reveal living cells yet some living cells would survive the heating and be present in the remaining unexamined portion. The impracticability of making a bacteriological analysis of each of 67,500 cc. is at once apparent.

In large scale efficiency tests of pasteurizers the amount of milk which can be subjected to examination is naturally limited and the results if positive, that is if the test organisms survive, of course show that the temperature is too low. If the results are negative, that is, show no evidence of living cells of the test culture, then the only safe interpretation is that there are none present in the amount of milk examined. Nothing can be said

of the rest of the milk in the test. It is because of the wide range between the "majority" and "absolute" thermal death-points of bacteria that this situation arises.

In view of these facts it is obvious that the selection of an effective pasteurizing temperature must be based, as it has been, on the "absolute" thermal death-point of pathogenic organisms determined under laboratory conditions. Large scale efficiency tests are unnecessary and are likely to be misleading. Knowing the "absolute" thermal death-points of pathogenic organisms the most valuable work for the future, in connection with pasteurization, will be a study of temperatures obtained in a commercial practice and the development of suitable instruments for determining that all the milk in a given pasteurizing process is heated to a point which will provide a safe margin above the "absolute" thermal death-point of pathogenic organisms.

SUMMARY

1. The wide range between the "majority" and "absolute" thermal death-point of a culture of *B. aerogenes* is pointed out.

2. The importance of difference between these thermal death-points is discussed in relation to large scale efficiency tests of pasteurizing processes.

3. It is shown that positive results, which are based on the survival of the test organisms are of value in efficiency tests, but that no reliance can be placed on negative tests which show the absence of the test organism in the relatively limited amount of milk which can be conveniently examined.

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STUDIES ON PASTEURIZATION[1]

XII. CAUSE AND SIGNIFICANCE OF PIN-POINT COLONIES FROM PASTEURIZED MILK

S. HENRY AYERS AND WM. T. JOHNSON, JR.

From the Research Laboratories of the Dairy Division, United States Department of Agriculture

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The appearance of so-called pin-point colonies when pasteurized milk is plated has led to much speculation as to what type of organisms they represent. In fact the frequent appearance of the colonies from the pasteurized milk of certain dairies has so disturbed health officials that there has been, in one instance, a disposition to prevent the sale of milk from these dairies. The cause of pin-point colonies is therefore important both to health officials and milk dealers.

PIN-POINT COLONIES

Before taking up the cause of pin-point colonies it must be realized that colonies of almost any organism which forms relatively small colonies may become pin-point in size if there are large numbers of them on a plate. Colonies are called pin points when they are just visible to the naked eye and appear very small under a hand lens. Suppose that a sample of milk is plated using a dilution of 1:100. If the milk contains 15,000 bacteria per cubic centimeter then there will be 150 colonies on the plate and the colonies will appear in their normal size. On the other hand if the milk contains 150,000 bacteria per cubic centimeter then there will be 1500 colonies on the plate and the chances are that a large proportion of them will appear as pin-point colonies. This is due to a restriction in their development by overcrowding.

[1] A list of other papers in this series is given at the end of this paper.

It is a general custom in control work to plate 0.01 cc. of milk when examining pasteurized milk, and under normal conditions this is a satisfactory dilution. If however the count increases, this dilution will not be great enough and through overcrowding of the plates the colonies may become pin-point in size. The relation of this practice of using one dilution of 0.01 cc. to pin-point colonies will be discussed later in this paper.

It is assumed in this discussion that counts are made on standard extract agar with the proper reaction and incubated at 37°C. for forty-eight hours. If the reaction is too high or too low this in itself may cause colonies to become pin-point in size. Incubation temperatures below 37°C. may prevent the appearance of pin-point colonies and slight variations in the composition of the medium may also exert an influence on their appearance.

Yates (1923) has noted the appearance of pin-point colonies in plates from pasteurized milk in Kansas City, Mo., made with standard medium and incubation, and found that the reaction of the medium had some relation to the appearance of such colonies. He also pointed out that another factor came into the local situation, the extensive use of chlorin solution at about the time pin-point colonies were observed. In regard to this factor Yates came to the conclusion that his data pointed strongly to the fact that the presence of pin-point colonies was closely connected with the use of chlorin solutions on the farms and at milk plants. He felt however that his results were not sufficiently comprehensive to warrant definite conclusions.

HISTORY OF TROUBLE WITH PIN-POINT COLONIES AT A MILK PLANT

The trouble from pin-point colonies that was experienced at a milk plant came to our attention in January. At this plant the general method for pasteurizing was as follows: The course of the milk was from storage tanks through a clarifier over a preheater, then through a pasteurizing tank where it was heated to 145°F. and held for thirty minutes. After holding, the pasteurized milk flowed by gravity over a cooler into the bottling machine.

Three tanks of the holder type were used for pasteurizing and were in constant operation from approximately 7:30 a.m. to 3:30 p.m., a period of about seven hours.

It should be kept in mind that as one tank of milk was pasteurized and emptied it was refilled and the process repeated. The ordinary grade of milk was pasteurized until about 2:00 p.m.; then one of the pasteurizing tanks was filled with a *special* grade of milk to be pasteurized. An examination of the raw *special milk* on a certain day in February showed that the average count was 28,000 bacteria per cubic centimeter, while after pasteurization, cooling, and bottling three bottles showed 72,000, 140,000, and 90,000 bacteria per cubic centimeter respectively.

Another series of tests on this special milk was made with the following results:

SAMPLE	BACTERIA PER CUBIC CENTIMETER		
	Raw milk	Pasteurized in laboratory	Pasteurized in plant
1. In weigh can as milk from each shipper was dumped	18,000	2,500	
2. In storage vat	37,000	3,000	
3. In storage vat two hours	65,000	3,600	
4. Through clarifier	72,000	2,500	
5. Over preheater	84,000	4,200	
6. In pasteurizing tank	150,000	130,000	
Out of pasteurizer over cooler			140,000
Bottling machine 1			140,000
Bottling machine 2			180,000
Bottling machine 3			150,000

It will be noted that the samples of raw milk 1 to 5 inclusive showed low counts when pasteurized in sterile bottles in the laboratory, but sample 6 of the special milk when it reached the pasteurizing tank showed a high count which was practically the same after pasteurization in the laboratory. Furthermore the count of the milk, sample 6, pasteurized in the laboratory checked well with that of milk pasteurized in the plant. The colonies on the plates were of the pin-point type.

At this point it is well to mention and to keep in mind that the special milk was run into a pasteurizing tank which had been in use with the ordinary grade of milk for about six hours. The tank was not steamed previous to filling with the special milk.

A few days later, bacterial counts of the ordinary pasteurized milk from the pasteurizing vats were made on standard agar at 37°C., with the following results:

Bacteria per cubic centimeter

Vat 1	2,500	Vat 10	4,700
Vat 2	60,000	Vat 11	100,000
Vat 3	4,400	Vat 12	110,000
Vat 4	11,000	Vat 13	80,000
Vat 5	70,000	Vat 14	240,000
Vat 6	18,000	Vat 15	120,000
Vat 7	15,000	Vat 16	200,000
Vat 8	140,000	Vat 17	180,000
Vat 9	100,000		

The vat numbers refer to the three tanks as they were sampled in series. Therefore vat 1 would be the first tank filled early in the morning, and tank 17 would be one of the three tanks filled later in the day, towards the end of the pasteurizing run. It will be observed that these counts varied, but were generally higher toward the end of the pasteurizing run, when the tanks had been in use for several hours. Pin-point colonies were noted when the counts were high. Under ordinary conditions at this plant a vat run, as shown above, would give an average under 10,000 bacteria per cubic centimeter when determined by standard methods.

FURTHER STUDIES OF THE PLANT INFECTION

Observations made in our laboratories on the development of bacteria at high temperatures led to the belief that the high counts and the pin-point colonies could be attributed to thermophilic bacteria. We are not aware of anything in the literature which shows that thermophilic organisms grow during the pasteurizing process as normally performed. Jensen (1921), how-

ever, has advised that skimmed milk be cooled after pasteurization instead of being returned hot to the producer. He found that the length of time in transportation might be several hours and that the skimmed milk during that period might be above 50°C. (122°F.). Jensen found that under such conditions changes were produced in the skimmed milk through the growth of thermophiles. From plates made of pasteurized milk from the plant, a number of bacteria were isolated from pin-point colonies which we found grew readily at 62.8°C. (145°F.).

A special incubator was arranged to operate at 50°C. (122°F.) so that plates could be incubated at that temperature, and composite samples of the special milk were taken at the plant of

TABLE 1

Comparison of bacterial counts made at different temperatures of incubation

SPECIAL-MILK SHIPPERS	BACTERIA PER CUBIC CENTIMETER AT DIFFERENT INCUBATION TEMPERATURES	
	37°C. (98.6°F.)	50°C. (122°F.)
No. 1	76,000	0 in 0.01 cc.
No. 2	6,400	0 in 0.01 cc.
No. 3	230,000	0 in 0.01 cc.
No. 4	423,000	0 in 0.01 cc.
No. 5	78,000	0 in 0.01 cc.
No. 6	463,000	0 in 0.01 cc.
No. 7	6,900	0 in 0.01 cc.
No. 8	5,400	0 in 0.01 cc.
No. 9	8,300	0 in 0.01 cc.
Mixed milk after pasteurization at the plant	202,000	628,000

each shipper of special milk, together with a sample of the same milk after pasteurization. The samples were plated on standard extract agar and on milk-powder agar, and incubated at 37°C. (98.6°F.) for forty-eight hours and at 50°C. (122°F.) for twenty-four hours. Both media support the growth of the thermophilic organism.

Interesting results were obtained as may be seen from table 1. The samples of raw milk, when the plates were incubated at 37°C. (98.6°F.), showed both low and high counts, while those incubated at 50°C. (122°F.) showed no colonies when 0.01 cc.

of milk was plated. After pasteurization at the plant, however, the milk showed a very high count on plates incubated at 50°C. (122°F.). This was proof that thermophiles were present in the special pasteurized milk but not in the raw milk, at least not in 0.01 cc., the dilution used in plating.

It is of further interest to note that plates from milk shippers 4 and 6 showed high counts and pin-point colonies, although it is clearly evident that in this case the pin-point colonies were due to overcrowding and not to thermophiles, since none showed upon incubation at 50°C. (122°F.). This point has been mentioned earlier in this paper.

TABLE 2

Bacterial counts of plant samples on plates incubated at 50°C. (122°F.)

SPECIAL RAW MILK		SAMPLES TAKEN DURING PROCESS	
Shipper number	Bacteria	Source	Bacteria per cubic centimeter
1	0 in 0.01 cc.	Special milk storage vat	0 in 0.01 cc.
2	0 in 0.01 cc.	Special milk through clarifier	0 in 0.01 cc.
3	0 in 0.01 cc.	Special milk over pre-heater	0 in 0.01 cc.
4	0 in 0.01 cc.	Special milk in pasteurizing tank	36,300
5	0 in 0.01 cc.	Special pasteurized milk over cooler	80,000
6	0 in 0.01 cc.	Bottled special pasteurized milk	52,000
7	0 in 0.01 cc.	Ordinary pasteurized milk (1)	7,100
8	0 in 0.01 cc.	Ordinary pasteurized milk (2)	24,700
9	0 in 0.01 cc.	Ordinary pasteurized milk (3)	25,000
10	0 in 0.01 cc.	Ordinary pasteurized milk (4)	37,000

Further data showed clearly that infection of the special milk with heat-resistant types of bacteria took place in the pasteurizing vats, and our results given in table 1 confirmed this view. It was considered desirable, however, to take another series of samples of the special milk during the pasteurizing process, so as to include each step in the process, and to determine the presence of thermophiles by incubating plates at 50°C. (122°F.).

The results in table 2 confirmed our previous experiments and showed that the thermophiles were not present in 0.01 cubic centimeter samples of raw milk from shippers. Neither did they appear in the storage tanks after the milk went through the clarifier and over the preheater. They first appeared when the

milk was in the pasteurizing tank and were present to the extent of 36,300 per cubic centimeter. The sample was taken as soon as the tank was filled but before it was held for the thirty-minute holding period at 62.8°C. (145°F.). The special pasteurized milk on the cooler showed a bacterial count of 80,000 at 50°C. (122°F.). This increase was probably due to the growth of the thermophiles. The 50°C. (122°F.) bacteria count of the bottled special pasteurized milk was somewhat lower due probably to the effect of cold on the organisms. This point will be discussed later.

There can be no doubt that the high counts of the special pasteurized milk were due to infection in the pasteurizing tank by thermophilic organisms. As pointed out previously, the special milk went into a tank in which the ordinary grades of milk had been pasteurized. This tank had been in operation for about six hours and was not steamed previous to receiving the special milk.

As shown in table 2, the 50°C. (122°F.) count of the ordinary pasteurized milk ranged from 7100 to 37,000 bacteria per cubic centimeter.

THE ORGANISM CAUSING PIN-POINT COLONIES

Thirty-nine cultures were isolated from 5 samples of pasteurized milk taken at the plant, including the ordinary and the special grades of milk. Of these, 37 cultures were identical, which indicates that at this plant the trouble was due to a single type of organism. Since the same organism has been isolated from pasteurized milk from another dairy, it appears that the organism may be the common cause of so-called pin-point colonies in cases where the trouble is due to a thermophile.

We have termed this thermophilic organism *Lactobacillus thermophilus*, since it apparently has not been described. It has the following characteristics: Morphology, rod, average size 3μ long and 0.5μ in diameter. Cells vary in length from 1.5 to 4.5μ. Stains are irregular, and often show lightly stained bodies in the cells. Spores are not formed. The organism is Gram-positive. It is a facultative anaerobe, but grows best aerobically. Will grow from 30°C. (86°F.) to 65°C. (150°F.).

The optimum temperature for growth lies between 50°C. (122°F.) and 62.8°C. (145°F.). It grows with great rapidity at the pasteurizing temperature.

In litmus milk a slight acidity is developed when the culture is incubated at 50°C. (122°F.) for twenty-four hours. The maximum acidity reached is not over 0.4 per cent calculated as lactic acid. At 37°C. (98.6°F.) several days incubation is required to show the slight acid reaction. Milk is not peptonized and gelatin is not liquified.

The organism when grown at 50°C. (122°F.) for three days in infusion broth with 0.5 per cent of the test substance and with the medium adjusted to pH 7.5 gives the following pH values, which vary slightly with different cultures.

Test substance fermented		*Test substance not fermented*	
Glucose	5.4	Salicin	7.0
Lactose	5.8	Mannitol	7.0
Sucrose	5.4	Raffinose	6.8†
Starch	5.8	Inulin	7.0
Glycerol	6.6*		

* Seems to be slightly fermented.

† May ferment slightly upon longer incubation.

Two other cultures, among the 39 studied, were thermophiles but distinctly different from *Lactobacillus thermophilus*. One was a spore former which was strictly aerobic and fermented only the test substances glucose, sucrose, and glycerol. In milk after forty-eight hours incubation at 50°C. (122°F.) there was no change, but in four days milk was coagulated with an alkaline reaction at the top of the tube. This spore-forming organism does not grow rapidly in milk at the pasteurizing temperature, perhaps because conditions of oxygen tension are not suitable, as this organism is a strict aerobe. It is fortunate that such is the case, for if this thermophile developed as rapidly as *Lactobacillus thermophilus* it would cause a type of plant infection difficult to destroy because of resistant spores.

The remaining thermophile showed no spore formation and gave no evidence of being a factor in causing plant contamination. Both of these cultures were probably stray organisms and of no importance.

TABLE 3

Growth of Lactobacillus thermophilus at different temperatures, in terms of bacteria per cubic centimeter

INCUBATION TEMPERATURE	BACTERIAL CONTENT								
	At start, in cold milk	After fifteen minutes heating to reach incubation temperature	After incubation period						
			One-quarter hour	One-half hour	One hour	Three hours	Four hours	Six hours	Twenty-four hours
71.1°C. (160.0°F.)	650*		0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	
71.1°C. (160.0°F.)	655,000	4,200	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	
68.3°C. (155.0°F.)	600,000	330,000		2,330	100	0 in 0.1 cc.	0 in 0.1 cc.	0 in 0.1 cc.	
65.6°C. (150.0°F.)	201,000	210,000		76,000	85,000	530,000		7,100,000	
62.8°C. (145.0°F.)	0 in 0.1 cc.†				0 in 0.1 cc.	900	4,000	84,000§	
62.8°C. (145.0°F.)	350				1,700	22,000	86,000		
62.8°C. (145.0°F.)	473,000				2,700,000‡	23,700,000	35,000,000	53,000,000	
62.8°C. (145.0°F.)	620,000	570,000		480,000	860,000	9,300,000		26,800,000	
50.0°C. (122.0°F.)	148,000	174,000		220,000	257,000	6,700,000	16,400,000	23,000,000	
37.0°C. (98.6°F.)	109,000							219,000	9,400,000
30.0°C. (86.0°F.)	125,000							163,000	535,000

* Milk inoculated when at a temperature of 71.1°C. (160°F.).

† Light inoculation. None present in 0.1 cc.

‡ Bacteria count after two hours.

§ After six and one-half hours, count was 471,000.

GROWTH OF LACTOBACILLUS THERMOPHILUS IN MILK AT DIFFERENT TEMPERATURES

Lactobacillus thermophilus is not a strict thermophile, for it will grow slowly at low temperatures. An idea of the growth range of the organism in milk may be obtained from table 3.

A culture of this organism was inoculated into sterile skimmed milk which was then heated to the desired temperature for incubation. Samples were removed at frequent intervals and plates were made which were incubated at 50°C. (122°F.) for twenty-four hours.

As the results show, when the culture was inoculated into milk at 71.1°C. (160°F.) no growth was evident in a six-hour period of incubation. In one experiment a reduction was shown in bacteria in fifteen minutes heating. At 68.3°C. (155°F.) there was a constant reduction in the bacterial count and no evidence of growth. At 65.6°C. (150°F.) there was a reduction during the first one-half hour, then a rapid increase up to six hours, when the experiment ended.

Lactobacillus thermophilus will grow rapidly, therefore, at a temperature as high as 65.6°C. (150°F.). It grows more rapidly, however, at a temperature of 62.8°C. (145°F.), the common pasteurizing temperature, and equally well at 50°C. (122°F.). A number of experiments were made at 62.8°C. (145°F.) with both light and heavy inoculations. It is quite evident that with a light inoculation the count may rise to quite large numbers after six hours of heating at the pasteurizing temperature. Since in milk plants the pasteurizing runs may require a six-hour period, it is probable that some milk remains in the tanks long enough so that a considerable growth of the thermophile may occur. As the milk flows from a pasteurizing tank, therefore, the varying amount of milk left in it, containing variable numbers of thermophiles, will serve as a variable inoculation for the next lot of raw milk going into the pasteurizing tank.

From the table it may also be seen that the thermophile grows slowly at 37°C. (98.6°F.) and more slowly at 30°C. (86°F.). It grows well enough at 37°C. (98.6°F.) to be visible after forty-

eight hours incubation on standard extract agar and appears as pin-point colonies only when large numbers are on the plate. With 100 colonies on the plate the colonies are of medium size. Reference is again made to what was said previously about the influence of overcrowding on the size of the colonies.

THE ABSOLUTE THERMAL DEATH POINT OF LACTOBACILLUS THERMOPHILUS

Since the appearance of pin-point colonies in the milk plant under observation was due to a plant infection with a thermophilic organism, it was necessary to know its absolute thermal death-point.

TABLE 4

Thermal death-point of lactobacillus thermophilus

Heated to 71.1°C. (160°F.) in 2½ minutes	Not held	Not killed
Heated to 71.1°C. (160°F.) in 2½ minutes	Held 5 minutes	Not killed
Heated to 71.1°C. (160°F.) in 2½ minutes	Held 15 minutes	Not killed
Heated to 71.1°C. (160°F.) in 2½ minutes	Held 30 minutes	Killed
Heated to 76.7°C. (170°F.) in 2½ minutes	Not held	Not killed
Heated to 82.2°C. (180°F.) in 2½ minutes	Not held	Killed

It has been shown that the organism will grow at 65.6°C. (150°F.) but not at 71.1°C. (160°F.). It is killed by heating at 71.1°C. (160°F.) for thirty minutes, or at 82.2°C. (180°F.) for two and one-half minutes, as shown in table 4.

The most striking feature of these figures, from a scientific standpoint, is the close relation of the maximum growing temperature to the absolute thermal death-point.

From a practical standpoint the fact that the organism is not a spore-former and can be destroyed by heating to 82.2°C. (180°F.) is of great value.

A milk plant can be freed of infection with this thermophile by heating all equipment, with which milk comes in contact, to 82.2°C. (180°F.).

EFFECT OF COLD ON THERMOPHILE

During the studies of the trouble with pin-point colonies and high counts it was observed that while the count after pasteurizing was often high, it was frequently low after storage at refrigerator temperatures for twenty-four hours. The reduction in count was also demonstrated by laboratory experiments, as is shown in table 5. There was a marked decrease in the number when a pure culture of the thermophile was held at 4°C. (39.2°F.) for twenty-four hours.

It may be expected that milk will have a higher thermophile count directly after pasteurizing than it will have at the time of delivery, provided, of course, it has been refrigerated and not immediately delivered.

TABLE 5

Effect of low storage temperatures on Lactobacillus thermophilus

BACTERIA PER CUBIC CENTIMETER			STORAGE TEMPERATURE
Original count after inoculation	After incubation at 62.8°C. (145°F.) for six hours	After storage for twenty hours	
6,400	4,400,000	9,100,000	20°C. (68.0°F.)
8,000	6,500,000	2,800,000	4°C. (39.2°F.)

SOURCE OF LACTOBACILLUS THERMOPHILUS AND PRESENCE IN MILK FROM OTHER PLANTS

Numerous materials from dairy barns have been examined, and while thermophilic organisms have been isolated we have not encountered *Lactobacillus thermophilus*. This organism has however been isolated from raw milk, and 11 cultures so obtained were identical with those isolated from pasteurized milk. It seems probable that the organism may be generally present in raw milk, but in small numbers, which may increase under suitable conditions. The source of the organism is therefore not clear.

This organism has been isolated from pasteurized milk received from two plants, and it seems apparent that it is frequently present in pasteurized milk.

SIGNIFICANCE OF PIN-POINT COLONIES DUE TO THIS THERMOPHILE

Since the organism is present in raw milk in small numbers, and will develop rapidly at the pasteurizing temperature, it is evident that a cumulative infection of tanks and other dairy machinery may take place unless the cleaning and sterilizing process is performed each day in such a manner as to destroy the organism.

If the pasteurizing tanks are properly cleaned and are in perfect condition, without leaks into the insulating walls, and are thoroughly steamed and all parts heated to at least 82.2°C. (180°F.), there should be little trouble from this thermophile. Steaming of the cooler and bottler should of course be included. This sterilizing process must be in daily operation preferably just after the pasteurizing run.

The presence of this thermophile in pasteurized milk appears to indicate improper sterilizing of milk plant equipment. In the milk plant under discussion it seems possible that the process of sterilization was inefficient because of leaks in the pasteurizing tanks. Small leaks were found in the tanks, which probably permitted milk to flow into the insulating walls and reach spaces where it could not be subjected to a temperature sufficiently high to destroy the thermophile. A leaking vat is a factor to be considered in efficient sterilization.

From a sanitary standpoint, except as an indication of improper sterilization of equipment which may result in high counts, the presence of the organism appears to be unimportant. It has, to our knowledge, been consumed for a considerable period of time with no indication of harmful results. *Lactobacillus thermophilus* appears to be a harmless saprophytic nonpathogenic thermophile, which is probably always present in raw and pasteurized milk in variable numbers.

LACTOBACILLUS THERMOPHILUS AND OTHER BACTERIA WHICH MAY GIVE PIN-POINT COLONIES

Pin-point colonies, as has been shown, probably appear as such because of low dilutions and consequent high numbers of

colonies on plates. When plating in a routine manner a pasteurized milk running about 10,000 bacteria per cubic centimeter, a 1:100 dilution will give 100 colonies which will appear normal in size. But if the count should suddenly jump to 100,000 then 1000 colonies would appear on the plates and, as we have previously stated, the colonies would be likely to appear as pin-point types. This is just what will happen if the increased counts are due to *Lactobacillus thermophilus*, but it will also happen if the increased count is due to heat-resistant streptococci or other bacteria. Sudden increases in the number of heat-resistant organisms frequently occur in raw milk, and they too may, in routine work at low dilutions, appear as pin-point colonies.

It is of importance to know whether the appearance of pin-point colonies is due to thermophilic or to heat-resistant streptococci or to other heat-resistant organisms which are not thermophilic, because the methods of eradicating the trouble will be different.

If due to a thermophile, the trouble is likely to be in the plant. If due to heat-resistant bacteria, present in large numbers in the raw milk before pasteurization, it is usually necessary to go back to the producing farm to locate the trouble. It can be quite readily determined whether thermophilic or non-thermophilic heat resistant organisms cause the high counts and pin-point colonies. When high counts and pin-point colonies appear, a set of plates should be made and incubated at 50°C. (122°F.) for twenty-four hours. A count in this manner will at once detect thermophiles. Should no colonies or only a few appear at 50°C. (122°F.) it can be assumed that the high counts and pin-point colonies are due to non-thermophilic heat-resistant bacteria. This can be verified by making standard counts at 37°C. (98.6°F.) of the raw milk and of the same milk pasteurized in the laboratory in sterile containers. The presence of a high proportion of a non-thermophilic heat-resistant types will be shown by a low percentage destruction of the bacteria by pasteurization.

Several years ago a sample of milk was sent to these laboratories, which had turned sour while being held for several hours at the pasteurizing temperature. From this milk Dr. Rogers

isolated a streptococcus which resembled *Streptococcus lactis*, but which grew rapidly at 62.8°C. (145°F.). It is apparent therefore that other types of thermophiles besides the one described in this paper may cause trouble in milk plants.

SUMMARY

1. Pin-point colonies probably result from overcrowding on plates through the use of dilutions which are too low. The reaction of the medium may also be a contributing factor.

2. The appearance of these colonies from pasteurized milk from one milk plant proved to be due to a thermophilic organism which we have termed *Lactobacillus thermophilus*.

3. A complete description of this organism is given and it is shown that it grows rapidly at the pasteurizing temperature.

4. The thermophile is easily destroyed by heating to 82.2°C. (180°F.).

5. *Lactobacillus thermophilus* appears to be a harmless saprophytic non-pathogenic thermophile probably always present in raw and pasteurized milk, but in variable numbers.

6. Pin-point colonies may also be due to non-thermophilic heat-resistant bacteria. Methods are described for determining whether this type or the thermophile is the cause of pin-point colonies.

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A NOTE ON A SIMPLE METHOD FOR OBTAINING UNCONTAMINATED SAMPLES OF INTESTINAL CONTENTS FROM VARIOUS LEVELS WITH A DUODENAL TUBE

R. G. FREEMAN, JR., AND E. G. MILLER, JR.

From the Laboratory of Biological Chemistry of Columbia University at the College of Physicians and Surgeons, New York

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The following device has been found useful in studying the intestinal flora and contents at different levels, and as no note of a similar method could be found in the literature, we offer this suggestion as an aid in such work. The desiderata were: To be able to introduce the bucket of a duodenal tube to any given distance without danger of contamination at the higher levels, and to be able then to aspirate a sample of the contents which would represent the conditions obtaining at that level; it was also essential that the apparatus could be sterilized.

Collodion (U. S. P.) is diluted with an equal volume of ether, and thoroughly mixed. The mixture (about 10 cc.) is poured on a smooth glass plate which has been thoroughly cleaned with alcohol. This is allowed to stand for one minute, and the excess is then drained off. The film on the plate is allowed to dry in the air for ten minutes, and then detached from the glass. The membrane may be kept in water until used. The membrane is placed over the duodenal bucket (which should be of the Rehfuss type, with large longitudinal openings) and fastened tightly by means of a rubber band at the junction of the rubber tube with the metal. The whole can be sterilized by boiling.

The patient then swallows the bucket and tube in the usual way. When the bucket has reached the desired depth (as ascertained by means of a fluoroscopic examination), the membrane is ruptured by sharp pressure applied with a 20 cc. Luer syringe inserted in the proximal end of the tube. The contents may then be aspirated.

THE FUNCTION OF LAG IN BACTERIAL CULTURES

JAMES M. SHERMAN AND WILLIAM R. ALBUS

From the Research Laboratories of the Dairy Division, United States Department of Agriculture, Washington, D. C.

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The period of lag, or the latent period through which a freshly inoculated bacterial culture passes before entering its period of rapid growth, has given rise to much speculation. A general résumé of the subject, and of the various views held concerning its nature, is unnecessary here since it has been discussed at length by several previous investigators. An excellent review has been given by Chesney (1916) in a comparatively recent paper. Although some of the earlier investigators considered the lag to be the fault of the culture medium, the works of Barber (1908), Penfold (1914), and Chesney (1916)—in which it has been amply demonstrated that transplants made from rapidly growing bacteria give no lag—show definitely that the period of reproductive latency is due to a change in the bacterial cells themselves. This has been clearly brought out by Chesney, who has shown that lag occurs when transplants are made immediately after the period of logarithmic growth is past. These and other facts led Chesney to the conclusion, which is the generally accepted one at the present time, that lag is the expression of an injury received by the cells in their previous environment.

While such an explanation in all probability is substantially correct from a practical point of view, biologically viewed it is perhaps not entirely adequate. The growth curve of bacterial cultures, of individual animals (Robertson, 1908), or plants (Reed and Holland, 1919, and others), of colonies of fruit flies (Pearl, 1921), and of human populations (Pearl and Reed, 1920), are all of the same nature, and are apparently controlled by the

same biologic laws. It is perhaps not fair to say that the animal which has stopped growing, or the population which has ceased to increase, has suffered an "injury."

Certain observations have led us to view the period of lag in bacterial cultures from a somewhat different angle. We have reported elsewhere (1922) results which indicate physiological differences between young and mature bacterial cells. It has been shown that the cells of rapidly growing cultures are more sensitive to certain harmful agents than are mature cells. For example, an exposure of one hour in a dilute solution of sodium

TABLE 1

Bacteria per cubic centimeter

TIME AFTER INOCULATION	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	Original culture	After one hour in 5 per cent NaCl	Original culture	After one hour in 5 per cent NaCl	Original culture	After one hour in 5 per cent NaCl
Hours						
0	55,500	66,000	96,000	82,500	70,500	70,500
1	51,500	55,500	80,500	60,500	75,500	62,000
1¼					68,000	57,500
1½	58,000	36,000	90,500	41,000	72,000	42,000
1¾					89,000	37,000
2	83,000	29,500	143,000	33,000	108,000	40,500
2¼					158,000	50,500
2½	106,000	21,500	255,000	16,500		

chloride in distilled water, while causing little or no injury to cells of an old culture of *Bacterium coli*, will cause a marked mortality among the cells from a rapidly growing culture of the same organism.

The hypothesis presented itself, therefore, that since old cells and rapidly growing or young cells are physiologically different, old cells would have to assume the characteristics of young cells before the culture could enter the phase of rapid growth. In other words, during the latent period the mature cells undergo a rejuvenescence and become fitted for reproduction.

In table 1 data are given from three different experiments bearing on this point. Transfers were made from cultures of *Bacterium coli*, which had been grown in 1 per cent pepton one

week at laboratory temperature, to fresh 1 per cent pepton with a reaction of pH 7.0 which had previously been warmed to 37°C. This newly transferred culture was then incubated at 37°C. Immediately after inoculation plate counts were made on the culture and at the same time 1 cc. of this culture was transferred to a 5 per cent salt solution, which was held at 20°C. At the end of one hour plates were made of the salt solution and the results calculated to the 1 cc. of the broth culture added. This procedure was repeated at stated intervals, as shown in table 1. In testing the resistance of the cells to salt solution 1 cc. from the culture was suspended in 100 cc. of the 5 per cent NaCl.

The results given in table 1 show that immediately after inoculation from an old culture, and for a period of an hour or so thereafter, the cells are not sensitive to the action of the 5 per cent NaCl solution, but after about two hours, when active reproduction is taking place, there is marked destruction of the cells when subjected to the same treatment. This is as was to be expected from our earlier work, but the point of the present paper is brought out by an examination of the results obtained when the cultures were one and one-half hours old, just before the end of the lag period. No measurable increase had taken place in the cultures at this period, but at the same time the salt treatment indicated a destruction of the cells from these cultures to a degree well beyond the probable experimental error. In other words, the mature cells from the old cultures assumed the characteristics of young cells before reproduction began. This fact, we believe, justifies the view that during the lag period the old cells undergo a biologic rejuvenescence which fits them for reproduction.

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THE VAN SLYKE METHOD FOR THE DETERMINATION OF AMINO-ACID NITROGEN AS APPLIED TO THE STUDY OF BACTERIAL CULTURES

R. W. LAMSON

From the Department of Bacteriology and Immunity, Harvard Medical School

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An increasing number of bacteriologists are devoting themselves to a study of bacterial metabolism in some one of its phases. In general, they have studied the products of bacterial growth or enzyme action on carbohydrate or protein media. This of course involves the use of many chemical methods. The average bacteriologist has not sufficient chemical training or the time to attempt to originate these methods, so that it has been necessary to use such existing methods as seemed suited to one's problem.

In following the changes in a protein medium the variation in amino-acid nitrogen is conceded to be a valuable index. The two methods which have been most used to determine this are Sorensen's (1908) formol titration, and Van Slyke's (1909, etc.) nitrous acid method. The Folin (1922) colorimetric method was used recently by DeBord (1923) with satisfactory results. The Van Slyke method has been the one most frequently employed. Dernby (1918) who had previously used Sorensen's method, considered Van Slyke's method the more satisfactory of the two. Avery and Cullen (1920) studying the proteoclastic enzymes of the pneumococcus, employed Van Slyke's method. An examination of their results indicates that satisfactory checks were obtained.

The writer selected the above method, using the micro-apparatus, for a study of the proteoclastic enzymes of *Salmonella schotmülleri* (*B. paratyphosus. B*) An associate also, tentatively, selected the Van Slyke method for amino nitrogen in his study of bacterial metabolism. Several hundred determinations were

run on pepton solutions, and the results in general were very irregular. Various changes in technique such as increasing the speed and time of shaking, were suggested by those more familiar with this method. These modifications and many others were applied without material improvement in the results. Many foam-inhibiting substances such as amyl alcohol, caprylic alcohol and diphenyl ether were used. The caprylic alcohol, when distilled according to the method used by Bock (1916) seemed to give the best results. DeBord (1923) finally abandoned the Van Slyke method, and states:

> Control analyses could not be obtained on a sterile 1 per cent pepton solution when this method was used. That the failure to obtain consistent control analyses was not due to the apparatus or reagents was shown by the fact that consistent blanks were obtained by myself and were checked by another worker in the laboratory. The variation in per cent in one particular series of tests was 18.4. Other series gave similar results and occasional tests showed errors even higher than the one indicated.

It was noted that if four to six determinations were made, two results might be obtained which would agree quite closely. This method of obtaining checks was adopted for a preliminary study.

The organisms were grown in extract broth, and the culture filtered through a diatomaceous filter. Such a filtrate was added to various solutions with a view of testing for enzyme action. As a control 10 cc. of filtrate was added to 50 cc. of salt solution. Another 10 cc. portion was added to 50 cc. of 1 per cent "Aminoid" pepton solution. A third 10 cc. portion was added to 50 cc. of 1 per cent "Difco" pepton solution.

As a preliminary step in the study it was necessary to know the content of amino-acid nitrogen in each mixture before incubation. The attempts to obtain such data are given in the following tables. The temperature of the room was noted frequently and was found to be constant during the time required for making the analyses—about two hours. Two cubic centimeters of the sample was used in each case. Caprylic alcohol

purified as indicated above was used as the foam inhibitor. The blank on all reagents including the foam inhibitor, was frequently determined and checks readily obtained. The amount of gas,

TABLE 1

Van Slyke amino nitrogen determination on 2 cc. of a salt solution—culture filtrate mixture

SAMPLE NUMBER	GAS	AMINO N PER 100 CC.	DEVIATION FROM MEAN
	cc.	mgm.	mgm.
1	0.09	2.56	−1.72
2	0.26	7.41	+3.13
3	0.07	1.49	−2.79
4	0.26	7.41	+3.13
5	0.12	3.42	−0.86
6	0.24	6.84	+2.56
7	0.08	2.28	−2.00
8	0.08	2.28	−2.00
9	0.18	5.13	+0.85
10	0.14	3.99	−0.29
Mean		4.28	

TABLE 2

Van Slyke amino nitrogen determination on 2 cc. of an "Aminoid" pepton culture filtrate mixture

SAMPLE NUMBER	GAS	AMINO N PER 100 CC.	DEVIATION FROM MEAN
	cc.	mgm.	mgm.
1	2.23	63.3	−3.94
2	2.35	66.6	−0.64
3	2.34	66.3	−0.94
4	2.38	67.5	+0.26
5	2.26	64.1	−3.34
6	2.44	69.1	+1.86
7	2.54	72.0	+4.86
8	2.37	67.2	−0.04
9	2.32	65.7	−1.54
10	2.49	70.6	+3.36
Mean		67.24	

usually 0.15 cc. was deducted from each test result and the final volume is reported in the tables. The technique was standardized in every way possible. The number of oscillations of the

deaminizing bulb was frequently noted and found to be constant at 250 per minute. The deaminizing bulb was shaken for five minutes and the Hemple pipette for three minutes, at a slower speed of course, and, in each case a second gas absorption was tried, but without materially changing the volume of gas.

The actual amount of nitrogen in each case in the above series is of course small and any fault in the technique would be greatly magnified. Such enormous variations indicate that this method is of doubtful value when such a mixture is to be tested.

TABLE 3

Van Slyke amino nitrogen determinations on 2 cc. of a "Difco" pepton culture filtrate mixture

SAMPLE NUMBER	GAS	AMINO N PER 100 CC.	DEVIATION FROM MEAN
	cc.	*mgm.*	*mgm.*
1	1.03	29.3	−3.08
2	1.18	33.6	+1.22
3	1.15	31.8	−0.58
4	1.22	34.7	+2.32
5	1.15	31.8	−0.58
6	1.19	33.9	+1.52
7	1.11	31.6	−0.78
8	1.16	33.0	+0.62
9	1.12	31.9	−0.48
10	1.13	32.2	−0.18
Mean		32.38	

The results obtained above, in tables 2 and 3, are not as variable as those given in table 1 and it seems worth while to subject them to a further statistical study. It is known that in any series of direct measurements, in spite of the most careful technique, the values will, in general, not be the same. Such indeterminate errors as produce the differences in the observed quantities follow the law of chance. One may ask what figures in such a series should be considered as most nearly representing a numerical measure of the reliability of the results. The most probable value of this quantity is given by the arithmetical mean of the series,—this has been determined in tables 2 and 3. As the mean has a higher degree of probability than any single ob-

servation from which it is computed, it is evident that the reliability of the mean increases with the number of observations. This leads one to consider how many observations should be made. It can be shown by the theory of probability that an arithmetical mean computed from n equally probable observaitions is $\sqrt{n}$ times as reliable as any one observation. Thus to double the reliability the observations must be fourfold, to treble it ninefold etc.

In any series of measurements taken under similar conditions, it sometimes happens that an observation will differ rather widely from others in the series, and the tendency to regard such an observation as erroneous and to reject it is very great. A good criterion to follow in such cases is the following: Compute the mean, m, and the average deviation, ad (the arithmetical mean of the numerical deviations, the sign of course being disregarded)—omitting the doubtful observation. Compute also the deviation, d, of the doubtful observation from this mean, m. If d equals 4 ad reject the observation. This criterion was applied to a few of the observations recorded in tables 2 and 3, but it was unnecessary to reject any of them. Several other tests were applied to these two series of results and in each instance the results were shown to follow the law of chance and therefore must be included if one is to attempt to measure the reliability of any single observation.

In table 2 it will be seen, if the sign is disregarded, that in six samples the deviation from the mean is between 0 and 2 mgm.; in three samples the deviation is between 2 and 4 mgm. Subjecting the results in table 3 to a similar analysis one finds eight samples whose deviation is between 0 and 2 mgm. and the remaining two samples between 2 and 4 mgm.

One also finds in tables 2 and 3 several pairs of results which are in quite close agreement, and had they been obtained as two out of three observations, many investigators would have disregarded the third observation. Thus, in table 2 samples 1 and 5; and 7 and 10 show similar figures, but it will be noted that the average of each pair of results differs by a very significant amount.

This figure alone might easily lead one to conclude that there had been a change in the amino-acid nitrogen content of a medium.

Sturges and Rettger (1922) using the Van Slyke method on a solution relatively poor in amino nitrogen state: "The possibilities of variation could never be sufficiently reduced, however, to place much reliance on a single determination. At least two determinations were always made, and when these gave results differing from each other by more than 10 per cent, the process was repeated until satisfactory checks were obtained." In only one instance do they indicate the amount of amino nitrogen with which they were working. In that case it covered a range of from about 4 to 85 mgm. per 100 cc. The lower figure indicates a range of nitrogen similar to that in table 1, and it is not surprising that these workers were unable to obtain entirely satisfactory results.

Wagner et al. (1924) also decided that the Van Slyke method was the one best suited to their problem. That the method was not entirely satisfactory in their hands is indicated by the following comment: "Occasionally in amino acid rich material such as is used in this experiment, as many as four determinations were made before satisfactory checks were secured."

DISCUSSION

It is evident from the results given in table 1 and the references cited that the Van Slyke method on the solutions indicated frequently yields very irregular results when the amount of amino nitrogen is relatively low. It would seem that the method is unsuited to such solutions, even if several samples are analyzed in an attempt to obtain satisfactory checks.

When the amount of amino nitrogen ranges from 30 to 70 mgm. per 100 cc. more satisfactory results may be expected. The averages of two sets of paired results obtained in ten consecutive observations on the same sample may differ from each other by a significant amount. In some instances this is large enough to suggest a change in the amino nitrogen content of the medium. If the mean of such a series of results is determined

the individual variations from this mean are in accord with the law of chance. This would indicate that the errors are indeterminate ones, and therefore the results are a measure of the probable reliability of the method when applied to the solutions previously described. If this method is to be used for the analysis of such solutions it is suggested that numerous portions be tested and the mean determined. Conclusions would not be justified unless a very positive difference was obtained between the mean of the control and test series.

The data reported in this study have received partial or complete confirmation by the other investigators cited above, and, on the whole, it appears that the method is far from satisfactory.

Such results indicate very strongly the dependence of this phase of bacteriology on the related subject of chemistry. We must look to the chemist for the development of new methods or the adaptation of existing ones to the needs of such problems as have been considered in this paper.

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THE OSTWALD VISCOSIMETER FOR THE DETERMINATION OF THE LIQUEFACTION OF GELATIN BY BACTERIA

FREDERICK W. SHAW

School of Mines and Metallurgy, University of Missouri, Rolla

Received for publication November 8, 1923

In a recent paper by Levine and Carpenter, there is a plea for a standardized method for ascertaining the change in viscosity of gelatin culture media. They suggest the use of Ostwald's viscosimeter, and that the readings be made at 40°C. It was later shown by Levine and Shaw, at the meeting of the Detroit Section of the Society of American Bacteriologists, October 5, 1923, that within certain limits of time and temperature of storage the viscosity of gelatin will give a constant reading at 40°C., irrespective of the history of the gelatin.

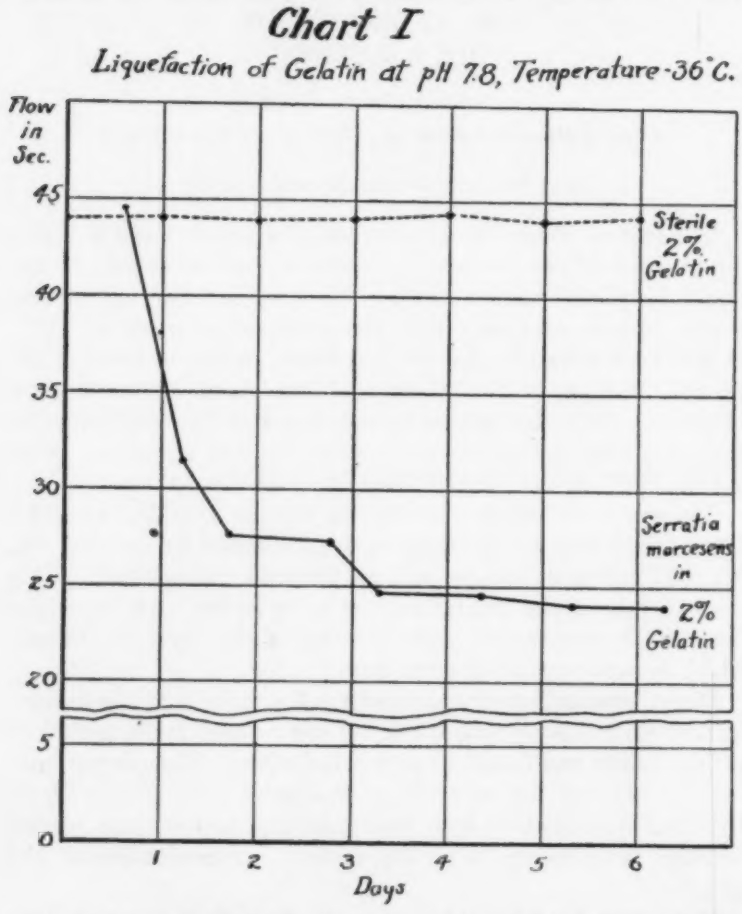
The work cited above was done by the use of ordinary culture tubes. The viscosity readings were preformed by pouring the gelatin solution or culture into an Ostwald viscosimeter. This method caused the gelatin culture to be useless after one reading, and it also caused contamination of the gelatin cultures which interferes with long readings.

Direct inoculation of sterilized gelatin culture media in Ostwald's viscosimeters was tried, by the writer, for a period of three months and found to give satisfaction. The longest continuous reading for any one viscosimeter was twenty days. Readings were made at least once each day, and at times several readings were taken in a day without contamination of the gelatin.

Care must be exercised in the selection of the viscosimeter. A viscosimeter whose bore is too small, should not be used as such an instrument is apt to become clogged with a small clump of organisms, or with a small piece of cotton from the plugs.

PREPARATION OF THE VISCOSIMETERS

Pour 4 cc.[1] of the gelatin culture medium into Ostwald viscosimeter of about twenty-five seconds flow for water. This



gelatin culture should be of about two per cent gelatin of good quality. Plug the two openings with non-absorbent cotton for a distance of about 2 cm. Care should be used in fitting the

[1] This amount varies in different viscosimeters.

plugs, because if these are fitted too tightly, the flow of gelatin in the viscosimeter will be retarded and a false reading obtained in the faster viscosimeters. Sterilize in the usual manner at 15 pounds for ten minutes. Remove from the sterilizer, cool to 40°C. and read. Keep the tubes at 40° until a constant reading is obtained. The flow of distilled water between the marks on the viscosimeter at 40°C. should have been previously taken and recorded. This need be taken but once for each instrument at the desired temperature. Inoculate through the reservoir side of the viscosimeter, in the usual way. The cotton should be kept dry as liquid in the cotton will obstruct the passage of air.

For the reading of cultures which have been incubated at 37° or higher, it is not necessary to heat at 50° for ten or fifteen minutes as it is with cultures which have incubated at lower temperatures. It should be understood that the cultures are placed in the 40° water bath at once on removal from the incubator. Readings at 40°, made on the cultures removed from the 37° incubator and placed immediately in the 40° water bath, give constant readings in less than fifteen minutes.

Mixing of the gelatin culture may be done by sending a bubble or two of air through the gelatin via the capillary side of the viscosimeter. Bubbles which have formed on the top of the gelatin, on the capillary side, are generally due either to too rapid income of gelatin into the measuring chamber, or to air in the capillary tube between small amounts of gelatin. These bubbles can be removed by gently blowing down the capillary tube as above described. There should be no bubbles in the capillary tube at the time of reading.

The water bath used by the writer, is a De Khotinsky, electric heated, with thermo-regulator. Some difficulty was experienced, at first, with the reading of the viscosimeters because the sides of the water bath do not admit light. This was overcome by using a microscope lamp having a frosted bulb and a sheet metal housing. After removing the stand, the lamp is suspended above the center of the bath with the lower portion

TABLE 1

Viscosimeter incubated at 37°C.

	TIME AFTER REMOVAL FROM INCUBATOR	VISCOSITY FLOW (40°C.)
	minutes	*seconds*
Tube 4	2	7.2
	15	7.2
	30	7.2
	45	7.2
	60	7.2
	120	7.2
	240	7.2
Tube 7	2	8.4
	15	8.4
	30	8.4
	45	8.4
	60	8.4
	120	8.4
	240	8.4
Tube 3	2	21.2
	15	21.2
	30	21.2
	60	21.2
	120	21.2
	240	21.2

TABLE 2

Viscosity readings with unplugged, loosely plugged, tightly plugged tubes. Time of flow in seconds

TUBE NUMBER	UP	LP	TP
4	7.0	7.2	7.8
7	8.2	8.4	9.0
3	21.2	21.2	21.2
1	115.4	115.4	115.4

Symbols: UP, viscosimeters without cotton plugs; LP, viscosimeters with lightly placed cotton plugs; TP, viscosimeters with tightly placed cotton plugs.

of the lamp within about 3 cm. of the surface of the water. This arrangement gives satisfaction, as the lines on the viscosimeter may be plainly seen by the dispersion of the light through the water.

TABLE 3

(Graphic chart I)

Observations of sterile 2 per cent gelatin pH 7.8 using the viscosimeter as a culture tube. Temperature 36°

TIME	VISCOSITY FLOW
	seconds
At beginning	44.0
End of first day	44.2
End of second day	44.0
End of third day	44.2
End of fourth day	44.4
End of fifth day	44.0
End of sixth day	44.4

TABLE 4

(Graphic chart I)

Observations of Serratia marcesens in 2 per cent gelatin, pH 7.8, using the viscosimeter as a culture tube. Temperature 36°

TIME	VISCOSITY FLOW
hours	seconds
14	44.6
29	31.6
41	27.6
66	27.4
77	24.8
104	24.5
127	24.0
150	24.0
210	24.0

Note the constant viscosity at 41 and 66 hour readings and the second drop between 66 and 77. This phenomenom may be seen in table 1 of Levine and Carpenter's paper.

It will be noticed in table 2, that the difference in the readings between the three methods, viz., without plug, with plug loosely packed, and with plug tightly packed is within the experimental error in tubes of twenty seconds or more. A comparison

of these readings will show that the cotton plugs in the viscosimeters do not interfere too greatly, for practical purposes, with the flow of the gelatin.

COMMENT

The readings of gelatin liquefaction by the former methods have been almost worthless for comparative purposes. Attempts have been made to prepare gelatin culture media of specific per cent gelatin. This is the method used in all of the laboratory manuals with which the writer is familiar. This is nearly impossible because of the many factors entering into the composition of gelatin. Some brands of gelatin contain more gel than others. In the writer's laboratory, one brand of 4 per cent gelatin solution became solid over night at 22°C., while a 15 per cent solution made from gelatin of another brand did not gel in a week at the same temperature. The time that the gelatin is in the autoclave has its effects on the gelation of the gelatin. These conditions are so general that it seems that a viscosity standard should be worked out to replace the per cent gelatin standard now in use.

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CLOSTRIDIUM PUTRIFICUM

III. A COMPARISON OF STRAINS OBTAINED FROM COLLECTIONS IN THIS COUNTRY AND ABROAD

GEORGE F. REDDISH

Medical College of Virginia, Richmond, Virginia

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There is still a certain amount of uncertainty in the minds of many bacteriologists concerning the identity of *C. putrificum*. In the early work of Bienstock, impure cultures were used, but by 1906 he was working with pure strains and his descriptions at this time are those of his type strain. These descriptions appearing in the Annales de l'Institut Pasteur are the final criteria for our guidance in this work. In one specific character Bienstock is emphatic; namely, the non-fermentative property of *C. putrificum* on any sugar. Since practically all other anaerobes ferment glucose, the inability of *C. putrificum* to form gas from this sugar is one of its most outstanding features. However, in the face of these definite records of the specific characteristics of this peculiar anaerobe, many workers in this country and abroad have described cultures which differ in very many respects from Bienstock's type strain.

In two recent papers on this anaerobe (Reddish and Rettger, 1922, 1923) a detailed study of its more important distinguishing features was made, emphasizing the morphology, the cultural characteristics and three phases of its biochemical properties. An attempt was then made to establish the organism as a distinct species, and to compare our own stock strains, isolated by Sturges, with those of Bienstock as described in 1906. The results were sufficiently constant and conclusive. Hall (1922), also, in his recent classification of the sporulating anaerobes, gives to *C. putrificum* its proper place in the round, terminal spore group

which digest albumin and brain slowly, and which do not ferment glucose. This places *C. putrificum* in the class intended by Bienstock when he suggested a separate group in which to include this species. In his study, the only difference he makes between *C. putrificum* and *C. tetani* is in the pathogenicity of the latter. This is in marked contrast to the descriptions given by the British Medical Research Committee (1917), which places this organism in the group characterized as having subterminal spores, as being actively proteolytic and also fermenting several of the common sugars with gas production. Later (1919), this Committee refused to recognize the existence of such a species, and claimed it to be a mixture of *C. sporogenes* and *C. cochlearum* or *C. tertium*. It is consistent with good practice to uphold the type strain of any organism as given by the discoverer, provided it is shown to be in pure culture when described. Bienstock's 1906 paper gives him this priority.

In the present investigation, the object has been to determine in just what respects the various strains of *C. putrificum* from representative collections differ and in what respects they resemble the strain described by its discoverer. At the same time, it was hoped to bring out some definite criteria on which to base identification. If we are to accept Bienstock's organism, those strains not complying with his descriptions must be discarded, or at least given another name.

The following strains were studied:

1. *C. putrificum* Hygienic Laboratory from Hall No. 22.
2. *C. putrificum* Hall No. 38.
3. *C. putrificum* (Sturges).
4. *C. putrificum* Pasteur Institute. Strain "B". No history.
5. *C. putrificum* Pasteur Institute Strain "M". No history.
6. *C. putrificum* Pasteur Institute Strain "G". No history.
7. *C. putrificum* Kral Collection. Strain E. Primban (Plant).
8. *C. putrificum* Kral Collection. Strain Car. verrucosus (navel of child).
9. *C. putrificum* Kral Collection. Strain No. 3—Bienstock Zeisler, Berlin.

Kendall's cultures had been disposed of, and were not available for the present study. I was unable, also, to obtain cultures from the Lister Institute, because they do not consider the organism to be a distinct species.

After preliminary study, I was convinced that the cultures received from Hall, the Hygienic Laboratory, and our own stock strains were all similar, and they did, in fact, come from the same original cultures of Sturges. Therefore, I included but two strains, one of our own and one from the Hygienic Laboratory. A stock strain of *C. sporogenes* (obtained originally from the Army Medical School) was included in the series for control. For convenience, the characteristics of these eight strains will be considered collectively.

In many outstanding respects, the strains fall into two distinct groups: (1) the round, terminal spore group, which digests protein slowly and does not form gas from any sugar; and (2) the oval, subterminal group, which breaks down the various kinds of protein readily and ferments various sugars with gas and acid formation. We shall consider these groups as nos. I and II, respectively.

MORPHOLOGY

Group I is characterized by having round, strictly terminal spores on the end of long, slender rods, which in old cultures form filaments. The Pasteur Strain "B" and our own stock strains belong to this group; the other two Pasteur strains and all three of the Kral strains fall into the second group, having oval subterminal spores. The vegetative rods of those in group II are about the same size as the rods of *C. sporogenes*, and do not form filaments in old cultures. It is very apparent here that we are dealing with two distinct groups of organisms, one with round terminal spores and the other with oval subterminal spores.

STAINING

All strains are Gram-positive and stain well with ordinary dyes.

MOTILITY

All strains are motile.

COLONY FORM

The strains in group II were found to have sporogenes-like colonies, both on the surface and in deep shake agar tubes. The Pasteur strain "B" grows readily in the depths of shake agar cultures and the colonies grow up to within half an inch of the surface of the medium. They are woolly and have no outstanding distinguishing features. Surface colonies of Pasteur strain "B" are delicate and somewhat similar to those of *C. putrificum* (Sturges) (see Reddish and Rettger, 1922, 1923). There is little difference between the strains as to the depth colonies.

CULTURAL CHARACTERS

Egg-meat. The organisms in group I grow slowly in egg-meat medium, showing very little change within ten days. After this time, there is digestion of the meat and putrefactive odors develop. The Pasteur strain "B" resembles very closely our stock strains in this respect. The organisms in group II attack meat medium very readily, showing gas formation within twenty-four hours, a turbid supernatant liquid and a slight odor; after thirty-six hours, definite digestion of the meat takes place with the evolution of distinct putrefactive odors. In this respect, they resemble *C. sporogenes*, and were equally as active as the control *C. sporogenes* culture in simultaneous growths.

Milk. The group II strains digest milk readily under solid paraffin, showing definite signs of growth within 36 hours. The action takes place quite as rapidly as does that of the *C. sporogenes* control. The organisms in group I attack milk very slowly and show definite digestion only after two weeks. This difference in types is again very marked in ordinary culture media.

Broth. There is abundant growth of all strains in twenty-four hours, excepting the Sturges strains, in both plain and glucose broth.

Gelatin. All strains liquified gelatin, the Pasteur "B" and our own doing so more slowing than the oval sporing group.

BIOCHEMICAL REACTIONS

Fermentation reactions. Briefly, the fermentation reactions of these strains are as follows: the organisms in group I do not form gas from any of the 18 test substances used, when tested by the shake agar method. The strains in group II ferment with gas production all the sugars that *C. sporogenes* ferments. This is one of the most significant features of the present study. It shows in another way, that we have as stock strains in some of the most reliable collections in the world two very different groups of organisms all of which are supposed to be true to the type strain of Bienstock. The Sturges strains and the "B" strain from the Pasteur Institute coincide with the description given by Bienstock in this respect also.

Glucose-consuming power. Here again a marked difference between the two groups is apparent. The Sturges strain reduces glucose 0.07 per cent in four days, the Pasteur "B" culture 0.06 per cent in the same period, while the oval sporing group consume from 0.37 to 0.42 per cent in the same time under like conditions. *C. sporogenes* in this test consumed 0.49 per cent; the similarity to the above cultures in group II is striking.

Peptolytic property. The simple Sörensen test was used for determining the relative amounts of peptolytic action by these strains on plain broth. Readings were made after growth for four days at 37°C. The figures are not of much assistance in separating the groups. In fact the "B" strain from the Pasteur Institute gives a Sörensen figure only slightly lower than the members of the other group, while the Sturges strains showed a distinct delayed action on peptone and gave a much lower figure than the other cultures at the end of four days' incubation.

Pathogenicity. All strains were found to be non-pathogenic to guinea-pigs, rabbits, and white mice.

DISCUSSION

From these very simple comparisons, it is evident that we have two groups of anaerobes, all of which are labeled *C. putrificum.* It is inconsistent with good practice that this condi-

tion should continue, especially since there are on record in explicit terms the distinguishing features of the organism as given by its discoverer, and as clearly substantiated by Reddish and Rettger, with other detailed descriptions now on record. We must of necessity accept the organisms included here in Group I as *C. putrificum*, and discard those in group II. The members of the first group are the only ones resembling the original strain.

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THE FATE OF BAKERS' YEAST IN THE INTESTINE OF MAN AND OF THE WHITE RAT

LEO F. RETTGER, GEORGE F. REDDISH, AND JAMES G. McALPINE

From the Sheffield Laboratory of Bacteriology, Yale University

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This subject is one of considerable general interest. Yeast cells have often been observed microscopically in normal feces of both man and animals. Occasionally, too, viable cells may be demonstrated by the usual plating method, though in very limited numbers. Few experiments on the influence of yeast administration have been recorded in which bakers' yeast was employed.

Neumeyer (1891) showed that yeast may pass through the digestive tract without losing its fermentative property. He fed 8 grams of brewers' yeast to human subjects and 10 grams to rabbits and cats and recovered many of the cells alive from the feces. More recently Völts (1919) demonstrated that yeast cells remained alive in the enteric canal of dogs for more than six hours. After nine and one-half hours most of the cells were dead and about half of the original number digested. Völts also used beer yeast.

Bakers' yeast, as well as any number of so-called "wild" yeasts, frequently gain entrance into the body of man and animals. Furthermore, the more or less widespread use of bakers' yeast as a therapeutic agent has aroused new interest in the fate of yeast in the digestive tract and in the physiological response of the host to the ingested yeast. The present report deals chiefly with (1) the influence of the host upon the viability[1] of yeast cells, (2) the influence of yeast feeding upon intestinal bacteria, partic-

[1] The terms "viable" and "viability" are used throughout this paper to designate the ability of yeast cells to multiply in the media employed for them in this investigation, and have no reference to their leavening power in the production or manufacture of bakery products.

ularly the gas-forming and the aciduric types, and (3) the physiological action of pure bakers' yeast on mice, guinea pigs and rabbits, when introduced by the subcutaneous, intravenous or intraperitoneal route.

METHODS EMPLOYED

Samples of feces of the white rats were collected on thick absorbent paper placed over the floors of the cages. They were weighed and thoroughly mixed with saline solution in stout test tubes, with the aid of broken glass. For slide mounting, for plating and for the study of gas production definite dilutions were made from the original suspensions. The suspensions of human specimens and the dilutions were prepared in essentially the same manner.

Viability studies were made on malt extract agar having a hydrogen ion concentration of pH 5.5 to 5.6. This agar was prepared by the method described by Reddish (1919) and had the following composition:

Malt extract, powder, Difco	100 grams
Water	900 cc.

This extract agar was sufficiently acid to prevent or greatly retard the development of bacteria, and hence readily permitted the enumeration of yeast colonies.

All culture plates were incubated at 30°C. for forty-eight hours.

Gas production and the relative numbers of gas-producing organisms were determined by inoculating deep lactose agar (Veillon) tubes with definite amounts of the diluted fecal suspensions and incubating at 37°C. for twenty-four to forty-eight hours.

Direct microscopic examination of the diluted feces was made on slides in the following manner: Films were prepared in the usual way, dried, fixed with alcohol and stained by the Gram method.

The per cent of Gram-positive bacteria and the per cent of Gram-positive rods of the aciduric type among the total Gram-positives were determined.

DIET

Small rats employed in the first experiments were fed 4 grams of ground dog biscuit and 2 to 3 cc. of melted butter as the basal diet. Later large rats were used and given 6 grams of the dog bread and from 3 to 4 grams of butter. When 2 grams or more of yeast cake were fed daily the rats received correspondingly less dog biscuit.

The yeast-fed rats received from 0.2 to 3 grams of bakers' yeast daily. In the lactose feeding experiments 3 grams of ground, dried white bread were given in place of the dog biscuit, in order to reduce the protein in the food. The amount of lactose was 3 grams. In all of these experiments the ground dog biscuit or bread was thoroughly mixed with the melted butter to make a smooth paste. This paste was packed firmly into heavy glass dishes (small moist chambers) and placed before the rats each day at as nearly the same time as possible. The rats were supplied with only as much food as they could consume on a given day.

The human subjects subsisted on their customary diets. During the yeast administration periods they took 14, 28 or 42 grams of yeast cake daily, amounts approximately equivalent to 1, 2, and 3 ordinary yeast cakes respectively.

EXPERIMENT I

The chief aim was to determine how small an amount of yeast may be ingested that will result in the excretion of viable yeast cells. Ten rats and 1 human subject were employed. Of these rats 2 received daily 0.2 gram of bakers' yeast, 2, 0.5 gram, and 4, 1 gram. The remaining 2 were held as controls. The results are summed up briefly in the following paragraph.

No living cells were at any time obtained from the feces of the 2 control rats. All of the yeast-fed rats excreted viable cells within forty-eight hours after the first feeding. The total number of living cells excreted per day by the rats receiving 0.2 gram of yeast cake daily amounted in some instances to at least 3 to 4 million, and for the animals which had 1 gram yeast cake to 40

million or more. The figures varied, of course, among the individual rats and in the same rats from day to day. Two to three days after the yeast feeding was discontinued but very few yeast cells could be recovered from the feces. Feeding of smaller amounts of yeast was not attempted.

The 1 human subject employed in this experiment took one yeast cake daily for five days, or according to the viability count 70 billion living yeast cells. Within twenty-four hours 12 million viable cells per gram of feces were eliminated. The number dropped appreciably during the three following days. Three days after discontinuing the yeast feeding the viable cells had practically disappeared from the intestine.

EXPERIMENT II

This had as its main object the determination of the fate of yeast cells in the body and the influence of the yeast upon other intestinal organisms and upon the host. The following amounts of yeast cake were administered daily:

White rats	0.5, 1 and 3 grams
Human subjects	14, 28 and 42 grams

Six white rats, including 2 controls, and 2 human subjects were employed.

All of the subjects receiving yeast began to excrete viable yeast cells within twenty-four hours after the first ingestion and continued to eliminate them during the entire period of yeast feeding. The numbers of viable cells recovered from the feces varied within wide limits from day to day. As a rule the first high curve peak was reached in about twenty-four hours. After yeast feeding was discontinued the numbers of viable cells excreted daily rapidly decreased, and by the third day very few could be detected in the feces. Disappearance was as a rule complete at the end of seventy-two hours.

EXPERIMENT III

Six rats, including 2 controls, and 4 human subjects were employed. The amounts of yeast administered and the results are given in tables 1 and 2.

TABLE 1

Showing numbers of viable yeast cells in the feces of 4 human subjects after ingestion of definite amounts of yeast cake

DAY OF YEAST FEEDING	NUMBER VIABLE CELLS TAKEN	NUMBER VIABLE CELLS EXCRETED DAILY		NUMBER VIABLE CELLS TAKEN	NUMBER VIABLE CELLS EXCRETED DAILY	
		Subject A	Subject B		Subject C	Subject D
	millions	*millions*	*millions*	*millions*	*millions*	*millions*
Preliminary period (two days)	None	None	None	None	None	None
First day	140,000			210,000	None	None
Second day	140,000	3.1	0.6	210,000	39.0	15.6
Third day	140,000	0.02	1.6	210,000	27.0	0
Fourth day	140,000	2.5	0.3	210,000	126.0	0.76
Fifth day	140,000	1.5	16.5	210,000	2.4	1.9
Sixth day	140,000	0.24	0.44	210,000	189.0	0.6
Seventh day	140,000	127.0	0.6	210,000	116.0	
Eighth day	140,000	6.3	6.9	210,000	0.26	0.8
Ninth day	140,000	1.1	1.7	210,000	16.6	1.2
Tenth day	None	5.0	1.9	210,000	140.0	2.0
Eleventh day	None	0	0	None	0	0
Twelfth day	None	0	0	None	0	0

TABLE 2

Showing numbers of viable yeast cells in the feces of 4 white rats after ingestion of definite amounts of yeast cake

DAY OF YEAST FEEDING	CONTROL RATS			RATS FED 3 GRAMS YEAST CAKE				
	Number viable cells fed	Number viable cells excreted daily		Number viable cells fed	Number viable cells excreted daily			
		No. 1	No. 2		No. 1	No. 2	No. 3	No. 4
	millions	*millions*	*millions*	*millions*	*millions*	*millions*	*millions*	*millions*
Preliminary period (two days)	None	None	None	None	None	None	None	None
First day	None	None	None	15,000	None	None	None	None
Second day	None	None	None	15,000	590,000	1.2	4,000.0	416.0
Third day	None	None	None	15,000	61.0	3.6	214.0	0.15
Fourth day	None	None	None	15,000	92.5	1.9	459.0	0.2
Fifth day	None	None	None	15,000	2.9	85.0	362.0	3.0
Sixth day	None	None	None	15,000	1.2	10.0	63.0	4.0
Seventh day	None	None	None	15,000	6.0	189.0	555.0	2.0
Eighth day	None	None	None	15,000	0.5	33.0	119.0	3.2
Ninth day	None	None	None	15,000	0.024	30.0	324.0	1.4
Tenth day	None	None	None	None	4.6	4.0	130.0	0.9
Eleventh day	None	None	None	None	0	0.4	0.8	0.4
Twelfth day	None	None	None	None	0	0	0	0

For corroborative purposes the above experiments were followed by a short yeast feeding experiment in which four human subjects were employed and in which each subject received three yeast cakes or approximately 42 grams of yeast daily for seven days. The results were in the main similar to those obtained with the two previous subjects which took the same amount of yeast, and for the sake of brevity are not tabulated here.

INFLUENCE OF YEAST FEEDING ON THE CHARACTER OF THE INTESTINAL FLORA

During the early part of the investigation there appeared to be some evidence that the administration of bakers' yeast to white rats and to human subjects caused a change in the relative numbers of Gram-positive and Gram-negative bacteria in the feces, with perhaps a slight but distinct increase in the number of rods of the *B. acidophilus* type. However, as the work progressed these apparent differences were lost and the conclusion arrived at that yeast feeding, as conducted in this investigation, has very little, if indeed any, influence on the relative numbers of various types of bacteria in the lower reaches of the intestine.

DISTRIBUTION OF YEAST CELLS IN DIFFERENT PORTIONS OF THE DIGESTIVE TRACT FOLLOWING YEAST FEEDING

Six white rats were given 2 grams of bakers' yeast daily for five days, along with the basal diet. The ration was set before them at 11:00 a.m. each day, in only the amount that the rats were able to consume during the next twenty-four hours. At 8:00 a.m. on the morning following the last feeding they were etherized and the stomach and intestine removed. Portions of the contents of the stomach, of the small intestine midway between the stomach and of cecum were introduced into test tubes and converted into a 1:10 emulsion with saline solution. The morning feces of the 6 rats were similarly treated. Malt extract agar plates were prepared from the different emulsions, and the colonies enumerated. Direct total counts were also made in stained films.

Only the results obtained from 3 of the rats are given in the following table. They are fairly representative of the entire group.

INOCULATION EXPERIMENTS ON WHITE MICE, GUINEA-PIGS AND RABBITS WITH PURE CULTURES OF SACCHAROMYCES CEREVISIAE ISOLATED FROM BAKERS' YEAST

There were employed in this investigation 3 white mice, 8 guinea-pigs and 10 rabbits. The 3 white mice, 5 guinea-pigs and 5 rabbits were given subcutaneous injections of from 0.5 to 1 cc.

TABLE 3

Showing the distribution of yeast cells in the digestive tract

SUBJECTS	SOURCE OF MATERIAL	TOTAL YEAST COUNT	VIABLE YEAST COUNT
Rat 3	Stomach	<10,000	<10,000
	Intestine	470,000	<10,000
	Cecum	1,500,000	2,100,000
	Feces	94,500,000	5,300,000
Rat 4	Stomach	3,150,000	<10,000
	Intestine	9,450,000	<10,000
	Cecum	252,000,000	10,000,000
	Feces	271,000,000	38,000,000
Rat 6	Stomach	17,000,000	<10,000
	Intestine	44,000,000	<10,000
	Cecum	164,000,000	9,500,000
	Feces	226,000,000	82,000,000

<Indicates fewer than number given.

of pure live suspensions of yeast, the suspensions having a turbidity corresponding to 3 to 5 on the McFarland nephelometer scale. The 3 guinea-pigs received subcutaneous injections of 1 cc. of yeast suspension on one side of the median line and the same amount of plain saline solution on the opposite side. In all of these experiments, except those on 2 guinea-pigs, 3 white mice and 2 rabbits, rectal temperatures were taken and the weights of the individual animals were recorded.

The subjects were kept under close observation, and besides recording temperatures and weights an effort was made to follow the course of any local reactions that might take place at the

TABLE 4

Daily rectal temperatures and weights

Rabbits

	I. MALE		II. FEMALE		III. FEMALE	
	Temperature	Weight	Temperature	Weight	Temperature	Weight
Preliminary period:						
First day	100.6	2,485	100.8	2,343	100.0	2,049
Second day	100.8	2,585	100.8	2,390	101.0	2,093
Third day	102.2	2,533	101.9	2,418	101.4	2,077
Intravenous injection (1 cc.):						
First day	101.9	2,684	101.1	2,567	100.0	2,144
Second day	101.8	2,595	100.6	2,482	101.2	2,070
Third day	102.0	2,540	101.6	2,472	101.8	2,099
Fourth day	100.7	2,546	101.0	2,474	100.2	2,105
Fifth day	103.1	2,617	102.3	2,544	101.5	2,090
Sixth day	102.7	2,580	102.4	2,443	101.7	2,046
Seventh day	102.4	2,604	102.1	2,435	101.4	2,033
Fifteenth day		2,755		2,690		2,056
	IV. MALE		V. MALE		VI. FEMALE	
Preliminary period:						
First day	100.7	2,030	100.4	1,924	102.6	2,847
Second day	102.0	2,076	102.2	2,008	101.8	2,805
Third day	102.1	2,058	102.2	1,936	101.9	2,885
Subcutaneous injection (1 cc.):						
First day	102.4	2,099	102.2	2,048	102.3	2,907
Second day	101.0	2,122	101.3	1,988	101.4	2,890
Third day	101.2	1,900	102.2	1,942	101.6	2,905
Fourth day	101.0	1,965	102.2	1,905	101.2	2,918
Fifth day	102.8	1,934	103.0	1,960	101.7	2,903
Sixth day	103.0	1,965	103.6	1,900	102.8	2,938
Seventh day	102.2	2,004	102.4	1,890	102.4	2,948
Fifteenth day		2,043		2,079		Pregnant

sites of inoculation. Each inoculation experiment was preceded by a preliminary period of three or four days in which the animals were kept under the same conditions as they were after the yeast

injections were made. In order to conserve space, only the tabulated data in table 4 are presented herewith.

Except in 2 instances, there was no evidence of harmful action by the yeast when injected by the subcutaneous, intraperitioneal or intravenous routes, in suspensions of from 0.5 to 1 cc. In a few animals there was a slight increase in temperature after the injection; however, the fluctuations from day to day are fairly well within the limits of variations of normal animals. The excitable nature of laboratory animals, particularly guinea-pigs, will of itself cause some irregularity, as the least exertion or nervousness on the part of the animal will bring about some increase in temperature.

In every instance there was full or almost complete maintenance of the original weight of the animal; 2 of the rabbits increased in weight following the injections.

In only 2 of the 21 animals that were employed as subjects was there any visible local reaction at the site of inoculation. In 1 guinea-pig and 1 rabbit there developed a small subcutaneous nodule which in the guinea-pig soon disappeared, but in the rabbit persisted for some time without softening or coming to a head. By the end of the third week, however, the nodule in the rabbit had disappeared. The weights of both the guinea-pig and the rabbit remained fairly stationary throughout the experiment.

The above observations are in accord with the results of a number of earlier investigators. Falk (1886) showed, contrary to the claims of Bernard[2] and Popoff (1872), that beer yeast when injected by the subcutaneous, intravenous, intrapleural or intraperitoneal method in rabbits and dogs caused no disturbances. Falk worked with pure beer yeast cultures and appeared to have come to the conclusion that the results obtained by Bernard and Popoff can be explained on the grounds that the pathological conditions produced by these observers were those of ordinary sepsis caused by bacteria which accompanied the injected yeast. Gilkinet (1897) also claims to have demonstrated that subcutaneous and intravenous injections of yeast do not

[2] Cited by Falk.

result in any harm to the subjects, and that the yeast cells are soon destroyed. He also used purified brewers' yeast.

Similar observations were reported by Neumayer (1891) and Jona (1897). The former made subcutaneous injections of 1 and 2 cc. of yeast cultures into mice and guinea-pigs and observed no harmful reaction. Jona experimented with a yeast known as *S. apiculatus*. He injected large numbers of the yeast cells into rabbits by the subcutaneous, intravenous and intraperitoneal methods with very little or no apparent reaction on the part of the animals, even when large quantities of inoculum were used. He claims to have shown that the yeast cells injected into the peritoneal cavity of the rabbit do not enter into the general circulation.

The present investigation dealt only with pure saline suspensions of bakers' yeast which were isolated on the malt extract agar used throughout the yeast studies and grown in pure culture on such agar preliminary to the preparation of saline suspensions for inoculation purposes. The so-called pathogenic yeasts, which have received considerable attention during the last twenty-five years and which the senior author has at one time given some study (1904) have, of course, undisputed pathogenic properties, when introduced into living animal tissues, and must not be confused with ordinary bread and beer yeast.

SUMMARY AND CONCLUSIONS

Bakers' yeast, when administered by mouth, underwent a rapid and extensive destruction in the alimentary tract. Less than 1 per cent of the cells ingested escaped this destruction and appeared in the feces as living cells within twenty-four hours. After the discontinuance of yeast feeding both dead and viable cells rapidly disappeared from the intestine until by the end of three days relatively few, if indeed any, remained. Most of the cells eliminated were dead, but there were always comparatively small numbers of viable cells which remained and grew readily on malt extract agar.

No difference could be observed as the result of yeast feeding in the proportion of Gram-positive and Gram-negative organisms,

or in the relative numbers of Gram-positive rods of the acidophilus type as compared with all other intestinal bacteria: nor was there noted any influence of the yeast feeding upon the amount of gas production in Veillon tubes.

The injection of pure suspensions of living bread yeast in white mice, guinea-pigs and rabbits by the subcutaneous, intravenous and intraperitoneal routes failed to show evidence of injury, aside from the formation of a small firm nodule in 2 (1 guinea-pig and 1 rabbit) of the 21 animals employed. These nodules disappeared without suppuration or necrosis. The temperature of the different animals was affected very little, if indeed at all, and in every injection experiment the weight of the animals remained practically stationary or increased during the period of observation following the injection.

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A BACTERIAL DISEASE OF SILKWORMS

R. W. GLASER

From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey

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I. THE PROBLEM, MATERIAL AND METHODS

During each of two summer seasons (1922 and 1923) between 3500 and 4000 silkworms were hatched from eggs purchased in the spring of 1922 from a dealer who possessed a disease-free stock. The writer took great pains to keep his silkworms in a healthy condition; nevertheless approximately one-third of the animals died from an epidemic which started in two separate batches. The rest were saved by isolation, sterilization and disinfection, and by practicing general cleanliness in everything concerning the handling of the worms. In both seasons, the young worms emerged from the eggs during the early part of June and were placed immediately in sterile trays, battery jars, and aquaria. These were covered by pieces of window pane glass. It was found that by leaving about one-fifth of the top of the jar open, moisture will not collect, but at the same time enough is held within to maintain the mulberry leaves in a fresh state for nearly twenty-four hours. The trays and jars were cleaned daily and fresh selected[1] mulberry leaves introduced.

While small, the worms were handled with sterile camel's hair brushes and were treated in mass (500 to a tray or jar). After the first moult, they were segregated in lots of 100; after the second moult, in lots of 50; after the third moult, in lots of 25; and after the fourth moult, in lots of 10. By treating the worms

[1] Tender leaves, free from molds, bird and visible insect feces or other dirt.

in this manner the disease, when it appeared in the stock, could be best controlled.

Each season the infection appeared after the second moult, and the number of cases increased progressively in those cultures in which it was impossible to stamp out the disease, probably on account of early mass infection. All cultures were examined daily and as soon as a worm appeared to be acting in an unusual manner or as soon as one died, it was immediately removed and the entire culture was transferred to a fresh sterile container and given fresh food. Diseased and dead worms soil the food and jars to such an extent that constant vigilance is necessary in order to hold the number of cases in check, and in some of those cultures in which only one or two cases appeared, during the early part of the season, it was possible to eradicate the disease entirely. In other cultures where more worms died, especially when older, it was noticed that a number of individuals survived, pupated and produced moths capable of laying fertile eggs.

For the experiments to be described, third and fourth stage worms were always chosen. These were taken from cultures in which no disease had appeared up to the date when used, or from cultures in which only one or two cases had been found early in the season. Since the microörganism which causes the disease invades the circulation during the later stages of the disease, sterility tests were made on the blood. Negative bacterial cultures were considered only as evidence that no microörganisms had as yet gained entrance into the body cavity, but when taken in conjunction with the state of health of the stock from which these worms were derived, the conclusion was justified that the worms were normal. By the use of controls it was found that this form of reasoning proved correct.

In a series of infection experiments a third or fourth stage worm was inserted into each of a number of sterile 6-ounce, wide-mouthed bottles. One or two fresh mulberry leaves were then taken for each bottle, placed in a sterile Petri dish and covered with a sterile distilled water suspension of a twenty-four hour agar slant culture of the organism studied. The

excess liquid was then drained off and the leaves partly dried by placing them between two pieces of sterile filter paper. The leaves were then inserted into the bottles and were readily eaten. After the infected leaves were consumed, the worms received normal mulberry leaves daily until they died.

II. GENERAL DESCRIPTION OF DISEASED LIVING AND DEAD WORMS

The infection has been observed to occur after the second moult of the worms and up to the time when transformation to the adult stage is first taking place within the pupal envelope. During the early stages of metamorphosis, many individuals succumb and soil the cocoons, thus ruining the silk. The worms give so little warning that it is difficult to detect diseased individuals, especially when they are kept in stock batches. All worms may appear perfectly healthy during the evening, but an examination the next morning will reveal a number of dead. In the experimental bottles it has been possible to observe the general external aspect of the affection more closely, but even here it is sometimes difficult to determine the state of health of a particular worm. Nevertheless, we may characterize a number of external features apparent during the last stages of the disease.

The symptoms usually appear from two to five days after artifical infection has occurred, death ensuing either on the same day or the day following. The first indications of disease are loss of appetite and listlessness, often accompanied by the passage of soft, moist feces. When in this condition, it is only a question of a few hours to a day until death occurs. The worm becomes progressively more inactive, and soon dies hanging by the prolegs in a flaccid condition from a leaf or from the side of the bottle, or it may often be found dead at the bottom. When first dead, and while the tissues within are still in a more or less organized condition, the color of the worm is white, changing to a yellowish hue a little later. While in this condition, the worm can be handled without rupturing its skin. If put on ice immediately after death, further progress of the disintegrative changes may be arrested for twenty-four hours or more.

If left at the normal temperature, however, the color of the worm soon changes to a light brown and later to a dark brown. This is the final stage of disintegration and at this time the organs and tissues of the worm are in a state of complete disorganization and dissolution. It is now extremely difficult to pick the worm up without rupturing the skin. If the skin ruptures naturally or on handling, a dark brown, odorless liquid will ooze out. This liquid and the moist feces passed during the stages prior to death are full of the disease-producing organisms.

When dead but a short while, the worms have no odor. Although the microörganism concerned with the disease is a proteolytic form, an odor of putrefaction was never noticed around the worms nor in any of the culture media used. Naturally, after the worm has been dead a while, and other bacteria have multiplied, the worms smell like other decomposing animal material.

In so far as can be determined, the course of the disease and the postmortem changes of the experimentally infected worms are identical with the picture obtained in the spontaneous stock cases.

III. HISTOPATHOLOGY

Three fourth stage worms were chosen from 3 separate cultures in which no disease had appeared, and 3 in the same stage were taken from 3 separate cultures in which only one or two spontaneous deaths had occurred early during the season, eradication of the disease in these cultures seeming probable. Blood cultures from these 6 worms remained sterile and the organism in question was not recovered from the feces. The worms were then killed, each one cut into thirds, the parts fixed, and sections cut from each third.

A study of the sections of the 6 worms did not reveal any bacteria whatever in the fore, mid or hind gut, nor in any of the tissues. The various organs and tissues also appeared perfectly normal (plate 1, fig. 1).

A number of fourth stage worms were experimentally infected with the bacillus to be described later. This micro-

organism was isolated in pure culture from the blood of a living stock animal in the last stages of disease. Later bacteriological work demonstrated that it was identical with isolations from other cases. Some of the worms were fixed every twenty-four hours after infection and sections cut in order to study the progress of the disease. Symptoms began to appear in three or four days, death ensuing the day after the appearance of symptoms in the case of nos. 7 and 8. The others were fixed prior to death; nos. 7 and 8 after death. Blood cultures and cultures from the feces were uniformly positive as soon as the sypmtoms were pronounced.

A study of the sectioned material gave the following results:

Worms 1 and 2. Twenty-four hours after infection. No symptoms. Blood negative. Feces faintly positive. Sections: Few bacilli in different parts of gut.

Worms 3 and 4. Forty-eight hours after infection. No symptoms. Blood negative. Feces positive. Sections: Many bacilli in different parts of gut.

Worms 5 and 6. Seventy-two hours after infection. Symptoms. Blood positive. Feces positive. Sections: Lumen of fore, mid and hind gut crowded with bacilli. In fore gut crypts filled with bacilli which seem to be penetrating the tissue. It appears as if bacteria in fore gut have an effect on the membrana chitinosa. Often where bacteria are bunched next to the membrane, the latter seems to fade away and in such places the bacteria are found in the epithelium and muscular wall of the fore gut and in places in the body cavity spaces adjacent to the gut. In the mid and hind gut the peritrophic membrane seems to form at least a temporary barrier against the invasion of the bacteria into the intestinal epithelium. Some of the body cavity tissues have a cloudy-like appearance. This is especially noticeable in the fat tissue. The nuclei of the fat tissue are greatly hypertrophied.

Worms 7 and 8. Seventy-two hours after infection. Symptoms. Ninety-six hours after infection, dead. Color of no. 7, dark brown. Color of no. 8, light brown. Skin of both ruptured on handling. Complete lysis of the internal organs and tissues. No odor. The brown fluid was full of bacilli. Sections: Complete disorganization of the tissues excepting the skin. The tinidia of the tracheae also seem to be resistant. Microörganisms scattered everywhere throughout the disorganized tissues (plate 1, fig. 2).

It seems that worms just prior to pupation are more susceptible to the disease and die more quickly than younger worms. This is probably due to the fact that during the normal histolysis of the larval tissues paralleled by the histogenesis of the adult tissues, permeable or weak places occur through which the bacteria can more readily enter the body by way of the alimentary tract. During the changes accompanying metamorphosis one must be extremely careful to distinguish between normal histolytic changes and the lytic changes caused by the disease producing organism.

IV. ETIOLOGY AND BACTERIOLOGY

During the early stage of the work in the spring of 1922, a Gram-negative, motile, non-spore-bearing bacillus was isolated many times from the feces and blood of living individuals and from the brown fluid of dead worms. At the first appearance of symptoms it was often possible to isolate the organism in pure culture from the blood.

For experimental purposes strains from different cases were used. The microörganism employed for the first infections was isolated from a worm that died in the stock with symptoms characteristic of the disease.

The results of the first set of experiments performed on 6 healthy third stage worms were as follows: Symptoms appeared between two and five days with death ensuing on the same or the following day. The diseased living worms and the dead individuals had the same appearance externally and internally as the diseased living and dead stock worms. The causative organism was again isolated from the fluid of each of the dead individuals; as a matter of fact, it was the dominant form.

The experiment outlined above was repeated on 6 healthy third stage worms with a strain derived from the blood of a living stock animal in the last stage of disease. Symptoms in the experimental animals appeared between two and four days with death ensuing on the same or the following day. The diseased living worms and the dead individuals had the same appearance externally and internally as the diseased living

and dead stock worms. The causative organism was again isolated from the fluid of each of the dead animals.

Twelve control animals accompanied the 12 experimental cases. These controls all lived and transformed into moths.

Table 1 gives the results of an experiment with 10 fourth stage worms infected with a strain from a worm that died in the stock. In this experiment the blood of some, the blood and tissues of others, and the tissues alone of a third set were studied from a bacteriological and histopathological viewpoint. The table is self-explanatory and also verifies the more detailed study previously described under section III. The worms that were simply bled might have lived long enough to die naturally of the disease, but in the 5 cases recorded on table 1, so much blood was used for the tests that it was thought best to kill the animals.

Ten fourth stage control worms were treated in exactly the same manner as the above individuals. No symptoms developed, the cultures from the blood were uniformly negative, no bacteria were found in the gut nor in the tissues, and the various tissues appeared normal. By taking only enough blood for the necessary tests, an effort was made to save those worms that were simply bled. All animals so handled reached maturity.

In section I it was stated that in stock cultures in which many individuals died and where infection of the healthy was unavoidable some, nevertheless, survived and transformed into moths, indicating individual differences in resistance. Why then does one ordinarily obtain a 100 per cent mortality in the experimental infections? This difference is probably due to the dose. The stock animals may become naturally infected one or a number of times, but the number of microörganisms must be far below that in the experimental infections described. Naturally infected worms are able to overcome or suppress the propagation of smaller initial doses of the parasite.

A description of the type characters that were constant for the different strains isolated from experimental or spontaneous cases of disease follows:

TABLE 1

Results of the examination of silkworms in fourth stage, infected August 15

NUMBER OF WORM	TIME OF APPEARANCE OF SYMPTOMS	METHOD OF PREPARING MATERIAL FOR STUDY*	GENERAL BACTERIOLOGICAL RESULTS	GENERAL PATHOLOGICAL RESULTS
1	August 17 (evening)	Fixed for sectioning	Bacteria in gut. Invasion of tissues.	Cloudy appearance of tissues, especially fat. Hypertrophied nuclei
2	August 18 (morning)	Cultures made from blood	Cultures positive	
3	August 18 (morning)	Fixed for sectioning	Bacteria in gut. Invasion of tissues	Cloudy appearance of tissues. Hypertrophied nuclei
4	August 19 (morning)	Cultures made from blood. Fixed for sectioning	Cultures positive. Bacteria in gut. Invasion of tissues	Tissues in early stages of disorganization
5	August 18 (evening)	Cultures made from blood	Cultures positive	
6	August 20 (morning)	Cultures made from blood. Fixed for sectioning	Cultures positive. Bacteria in gut. Invasion of tissues	Cloudy appearance of tissues. Hypertrophied nuclei
7	August 19 (morning)	Cultures made from blood	Cultures positive	
8	August 19 (evening)	Fixed for sectioning	Bacteria in gut. Tissues not yet invaded	Tissues appear normal
9	August 20 (morning)	Cultures made from blood	Cultures positive	
10	August 18 (evening)	Cultures made from blood	Cultures positive	

*The worm was prepared for study as soon as symptoms appeared.

1. Morphology

Gram-negative bacillus. Twenty-four hour bouillon culture, bacillary forms dominant (plate 2, fig. 3). Twenty-four hour agar culture, bacillary and coccoid forms. Five day old agar slants, coccoidal forms dominant (plate 2, fig. 4). Size in length from twenty-four hour bouillon culture, 0.5 to 1.5μ; from nutrient agar 24 hours, 0.25 to 0.75μ. Formation of chains absent. Motile. Spores absent. Capsules present in twenty-four hours in milk and in nutrient bouillon.

2. Cultural characters

Nutrient bouillon: Twenty-four hours, 37°C. Cloudy, no ring, no pellicle, some sediment. Ring in forty-eight hours. No odor of putrefaction.

Agar stroke: Twenty-four hours, 37°C. White, abundant, glistening, smooth.

Potato: Twenty-four hours, 37°C. Abundant, white or with light yellow tinge.

Agar colonies: Twenty-four hours, 37°C. Round or irregularly round, diameter 0.3 to 4.5 mm.

Gelatin colonies: Forty-eight hours, 22°C. Punctiform. In four days, somewhat larger. Liquefaction.

3. Biochemical reactions

Milk: Reaction in twenty-four hours, 37°C. from pH 6.6 to 6.4. Rennet curd. Reduction of litmus in ten days. Peptonization of curd.

Gelatin stab: One week, 22°C. Liquefaction nearly complete.

Coagulated horse serum: One week, 37°C. Liquefied.

Blood agar: Twenty-four and forty-eight hours, 37°C. No hemolysis.

Relation to oxygen: Facultative anaerobe.

Diastatic action: Absent.

Indol production: Absent.

Voges-Proskauer reaction: Positive.

Table 2 gives the maximum acidity and change in acidity as well as the amount of gas development, in seventy-two hours, produced by the bacillus grown in bouillon containing 1 per cent of various carbohydrates.

During the course of the work 12 strains were isolated from spontaneous stock cases. These 12 isolations were fully studied morphologically, culturally and physiologically, and tallied except for insignificant variations in regard to changes in the hydrogen potential in sugar bouillon. With 7 of these isolations disease was produced; the remaining 5 were simply studied as above stated.

TABLE 2

Change in pH and gas produced by organism in 1 per cent sugar bouillon

	pH OF BOUILLON	pH PRODUCED IN SEVENTY-TWO HOURS*	CHANGE IN pH PRODUCED IN SEVENTY-TWO HOURS	GAS
				per cent
Glucose	7.3	6.6	+0.7	+52
Lactose	7.5	7.5	0	−
Sucrose	7.6	6.4	+1.2	+79
Maltose	7.4	5.9	+1.5	±4
Mannite	7.6	6.3	+1.3	±7
Dulcite	7.6	8.0	−0.4	−

*Maximum pH is produced at this time.

The bacillus does not produce any gas in fermented bouillon, showing that the gas obtained in some sugars is formed through the hydrolysis of the sugars and not from the proteins. Moreover, absorption tests with NaOH demonstrated that the gas formed was almost entirely CO_2. After this was removed only a small bubble remained in the closed arm of a Smith fermentation tube. In this respect, the behavior of the bacillus discussed reminds one very much of the activities of certain yeasts.[2]

[2] In the matter of CO_2 production the organism also slightly resembles *B. cloacae*. The latter organism, however, has the gas ratio $\frac{3(CO_2)}{1(H)}$ in contradistinction to the gas ratio of the silkworm organism which is $\frac{1(CO_2)}{0(H)}$.

V. THE QUESTION OF TRANSMISSION THROUGH THE EGG AND THE VIABILITY OF THE BACILLUS

It is often claimed that Pasteur proved that "Flacherie" is transmitted from generation to generation through the egg. Pasteur's work showed that pébrine, a microsporidian disease of the worms, could be transmitted by the moths to the succeeding generation. The term "Flacherie" is used to designate what we now suspect to be a diseased condition of silkworms, caused by a considerable group of bacteria which chance to enter the alimentary system. "Flacherie" is also entirely distinct from the polyhedral affection called "jaundice," "grasserie," or "gelbsucht." "Jaundice" has often been confused with "Flacherie" and also with "pébrine." Some reliable evidence exists to show that "jaundice" is also probably transmitted through the egg.

On reviewing Pasteur's[3] orginal work on "Flacherie," however, one is certainly not impressed with the fact that any evidence exists which proves that bacteria pass through the eggs of silkworms. Later workers in this subject have not been able to show any reasons for abandoning this point of view.

The decision as to whether the disease discussed in this article may be grouped under the "Flacherie"-like affections or not had best be "pigeon-holed" for the present. However, we are here dealing with a bacterial malady having the same external aspect as "Flacherie" and originating in the alimentary tract. On account of this close relationship, it was thought well to determine, if possible, whether the bacillus described could be found on the exterior or in the interior of eggs laid by the female silkworm moths.

The eggs used for the experiments were taken from stock cultures in which many worms had died and in which mass infection seemed certain. A few of the individuals in these cultures survived, however, and laid fertile eggs. If the bacillus enters the eggs it should be possible to find the organism.

[3] Pasteur, L. M. 1870. Études sur la maladie des vers à soie. La Flacherie. Tome 1er; pp. 207–317; planches 10.

If the bacillus does not enter the eggs it may, nevertheless, survive on the surface of the chorion for all eggs taken were soiled with the dried juices of worms that died of disease in the identical trays in which certain early moths had first deposited.

To determine these points, eggs four months old were used. Six eggs were crushed in each of two nutrient broth tubes without subjecting them to any previous treatment. Twelve eggs were submerged for five minutes in 80 per cent alcohol and 6 received the same treatment for ten minutes. They were then washed in sterile distilled water and transferred to bouillon in which they were crushed. Twelve eggs were submerged for two minutes in 5 per cent phenol and 6 eggs received the same treatment for five minutes. This was followed as before by the water washing, after which the eggs were crushed in bouillon. Six eggs were treated for two minutes in a solution composed of one-half by volume of 1:1000 dilution of $HgCl_2$ plus one-half by volume of 95 per cent alcohol, and 12 eggs received the same treatment for five minutes. This was again followed by washing in water and crushing in nutrient bouillon. All tubes were then incubated for seventy-two hours.

The disease-producing bacillus was not recovered either from the exterior or from the interior of the eggs. One of the tests with 80 per cent alcohol yielded a growth, but the bacillus in question was not found. The broth tubes in which the eggs were crushed without the previous sterilization of the exterior yielded growths, but subsequent work involving a study of four organisms recovered did not demonstrate the presence of the disease producing bacillus.

In connection with the general subject of the resistance of the bacterium to its environment, it might be instructive to outline one more experiment performed. Four stock tubes, representing 4 strains of the bacillus, were sealed with sealing wax and placed on ice for three months (October 15 to January 18) without transferring to fresh media. On January 18, the cultures were removed from the ice and subinoculations made into fresh media. These transfers were incubated for seventy-two hours. In only 1 slant out of the 4 did a growth appear. The other 3 remained sterile.

The experiments with the silkworm eggs as well as the one just described seem to show that the microörganism is not very resistant towards the detrimental effects of its surroundings.

VI. SUMMARY

A bacterial disease which rapidly assumed epidemic proportions appeared among the author's silkworm cultures. This disease was investigated and the methods used for its study and control described. The external appearance of diseased living and dead worms is described and an account of the histopathology of the malady is given. A bacillus was isolated from the feces, blood and various tissues of diseased living and dead worms. Etiological studies demonstrated that the bacillus is the causative agent. The investigations showed that dying and dead worms infect the food of normal silkworms by means of the feces and body fluids. The normal worms ingest the bacilli with the food. After multiplying in the alimentary tract, the bacteria penetrate into the circulation and into the organs, producing certain changes and rapidly causing complete histolysis of all the tissues. Symptoms appear in two to five days after experimental infection, death ensuing on the same day or the day following. Notwithstanding the great pathogenicity of the microörganism, close observations on all the silkworm stock cultures showed that a form of resistance towards the disease exists among some individuals.

A detailed bacteriological study was made of all microörganisms isolated from experimental and spontaneous cases. Out of 12 isolations made from spontaneous cases, the disease was reproduced with 7. The 5 remaining strains were not tested as to their pathogenicity, but were carefully studied culturally and biochemically. All of these isolations agreed in the morphological, cultural, and biochemical characters studied within the limits of reasonable slight variations.

Some evidence is advanced to show that the disease-producing bacillus is not transmitted from generation to generation through the egg. It is further shown that the bacillus is probably not

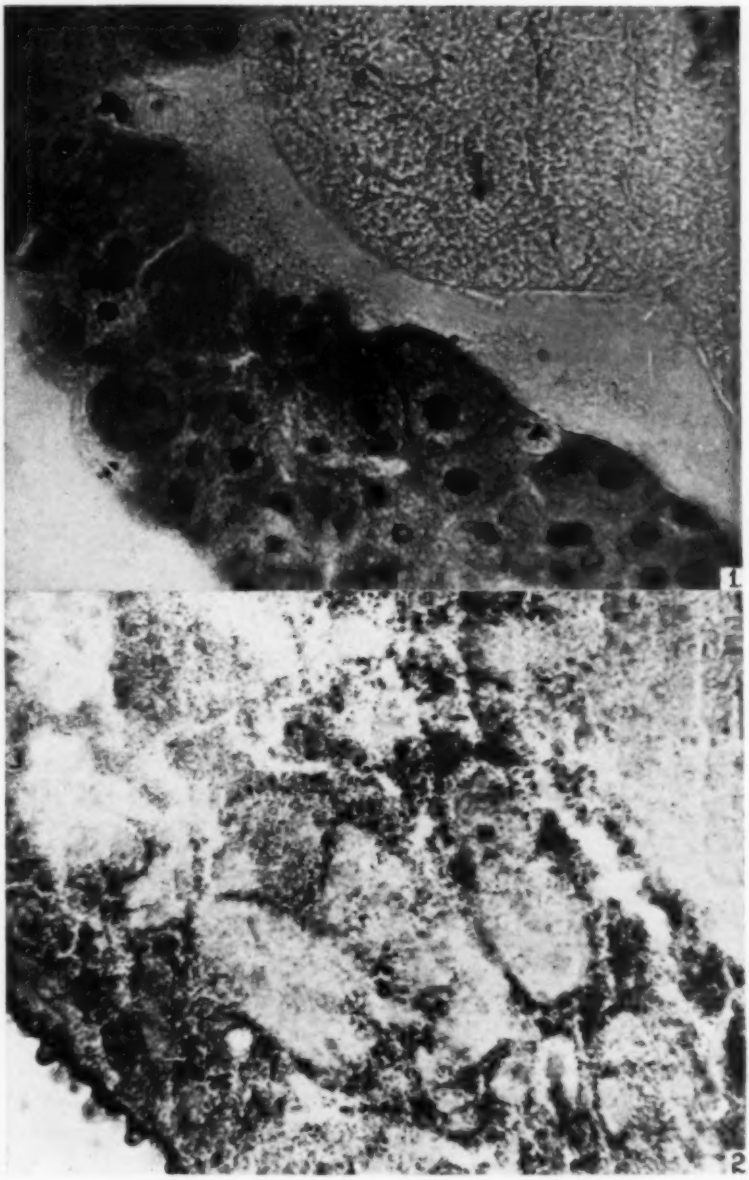
very resistant to its environment and does not survive for any great length of time on the exterior of the eggs.

The possible relation of this disease to the "Flacherie"-like affections of silkworms is mentioned and its distinctness from pébrine, muscardine, and the polyhedral disease, also termed "jaundice," "gelbsucht," or "grasserie" is emphasized.

PLATE 1

FIG. 1. Section through a part of the body cavity of a normal silkworm. Only a portion of the various tissues is shown. The muscle is cut crosswise showing fibrillar ends as dots. This is given above and to the right. Below and extending to the left side is shown a portion of the fat body. Some blood cells, coagulated serum, and a piece of a tracheal tube may also be seen. × 1000.

FIG. 2. Section through a portion of a silkworm fixed a few hours after death by disease. Complete lysis of the tissues excepting the skin has occurred, and enormous numbers of bacteria are scattered throughout. × 1000.



(Glaser: Bacterial disease of silkworms)

PLATE 2

FIG. 3. Disease producing bacillus from twenty-four hour bouillon culture. Some involution forms, but bacillary type dominant. × 1000.

FIG. 4. Disease producing bacillus from five day old agar slant. Coccoidal forms dominant. × 1000.

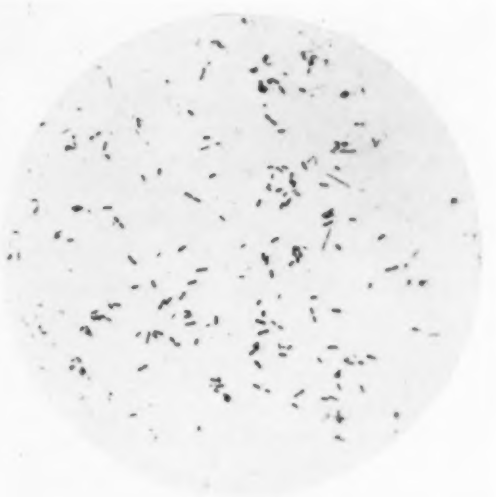


Fig 3

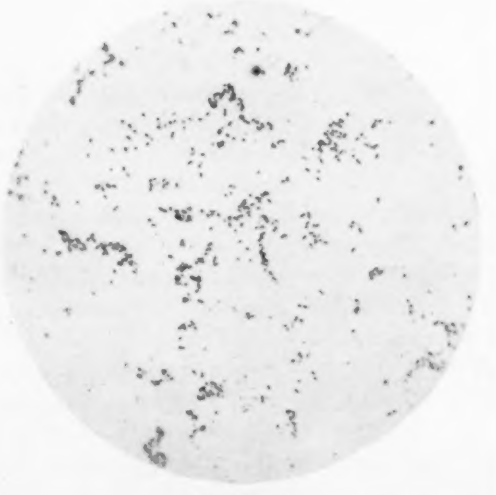


Fig 4

(Glaser: Bacterial disease of silkworms)

COMPARATIVE STUDY OF LACTOBACILLUS ACIDOPHILUS AND LACTOBACILLUS BULGARICUS

WALTER L. KULP AND LEO F. RETTGER

From the Laboratory of General Bacteriology, Yale University

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INTRODUCTION

For the past thirty years or more investigators here and abroad have given considerable time and thought to the study of the Gram-positive bacteria which make up the genus Lactobacillus (Committee on Classification, 1920).

Members of this genus comprise species which have been isolated from, and considered the most important fermentative agents in fermented milks from different parts of the world, as for example, mazun of Armenia, yogurt and kefir of Russia and the Balkans, leben raib of Egypt and gioddu of Sardinia. Members of this genus have received much attention in the study of the intestinal bacteriology of man and of many of the lower animals, the fermentation of ensilage and sauerkraut and the biology of salt-rising bread. They have been shown also to be a very important factor in the ripening of various kinds of cheese. At times their presence in soil has been noted; in fact they can as a genus be called almost "ubiquitous" bacteria.

This research deals with those members of the genus Lactobacillus which for the present may be considered as embodying three main types or species. The first of these is the organism found in fermented milks and called *B. bulgaricus* by Grigoroff (1905). The second is an intestinal bacterium, first isolated and studied by Moro (1900) and designated by him as *B. acidophilus*. The third species is another intestinal organism which is found particularly in the intestines of breast-fed infants and which was first studied by Tissier (1900) and by him given the name of *B. bifidus*.

The actual experimental work reported in this paper concerns the first two groups, *L. bulgaricus* and *L. acidophilus*. Such a study appeared important because of the recent work of Rettger and Cheplin (1921) on the implantation of *L. acidophilus* in the human intestine as a means of combating constipation, diarrhea and other intestinal disturbances. It is claimed by many that so-called auto-intoxication and general debility are caused by the absorption of accumulated products of bacterial putrefaction in the colon. Ever since Metchnikoff (1908) advocated the drinking of Bulgarian milk as an aid in securing longevity because of its apparent beneficial action upon the colon, there has been considerable interest in sour milk therapy, especially the use of *L. bulgaricus* milk.

Metchnikoff and his contemporaries thought the good effects of feeding Bulgarian milk were due to implantation and proliferation of *L. bulgaricus* in the intestine. More recently Rettger and his associates have shown that the beneficial influence results from increased growth of *L. acidophilus*, an organism native to the intestine and the growth of which is fostered in milk drinkers by the lactose contained in milk.

Without going further into a discussion of sour milk therapy, it will suffice here to note that the two opposing claims stimulated renewed interest in comparative studies of these two aciduric organisms. The generic term "Lactobacillus" has generally been used throughout this paper in accordance with the new classification adopted by the Society of American Bacteriologists.

In the present work the authors have attempted to acquire more complete first-hand knowledge regarding the characteristics of *L. bulgaricus* and *L. acidophilus*, particularly those which may be of importance in their differentiation.

GENERAL HISTORICAL REVIEW

For a complete historical review and bibliography, the reader is referred to the doctorate thesis of the Senior author in the Yale University Library.

A review of the literature covering the investigations of members of the Lactobacillus genus found in fermented milks, in the

intestines of man and animals, and of those obtained from other sources, reveals much confusion, especially in regard to nomenclature and identification. It appears that identical species isolated by different investigators from closely related sources have in many instances been given different names.

Grigoroff (1905), working in Massol's laboratory, reported two species which he had isolated from "podkvassa," the starter for the Bulgarian "kiselo mleko." His species "A" has been considered the type species of the Lactobacillus group and has been called *L. bulgaricus* (Massol).

Weigman, Gruber and Huss (1907) studied an organism isolated from Armenian sour milk which they named *Bact. mazun.* Heineman and Hefferan (1909) isolated a number of strains from various sources, which they considered identical with *L. bulgaricus.* Kindraczuk (1913) gave the name *B. carpathicus* to an organism similar to *L. bulgaricus,* which he isolated from "huslanka," a fermented milk of Bukowina and the East Carpathians.

Moro (1900) isolated an organism from the feces of breast-fed infants, the "Blaubacillus," to which he gave the name of *B. acidophilus.* Moro's organism was, as he supposed, derived from the mother's breast and was found in the mouths and stomachs of infants as well as in the intestinal contents. He stated that it was not a single species but a group of closely related forms which preferred an acid medium for growth.

Cahn (1901) noticed *L. acidophilus* in the feces of breast-fed and bottle-fed babies. Rodella (1901) isolated a similar organism from the same source and called attention to its pleomorphism. The organism varied in form from coccoid to very long rods in the same culture, some of the rods showing branches. Weiss (1904) noted that when large quantities of milk were ingested a marked increase of organisms of the lactobacillus type, corresponding to the *L. acidophilus* of Moro, was observed.

Tissier (1908) in a study of the intestinal flora of infants observed that at the age of about three days, breast-fed babies developed an intestinal flora in which one organism was

predominant. This had in general the staining reactions and morphological appearance of the lactobacilli. Tissier noted the presence of many bifurcated forms and, because of this characteristic gave his organism the name *B. bifidus*. Having isolated it, he discovered that it was anaerobic, in distinction from *L. acidophilus* which was facultative. This same worker also mentioned an organism, *B. exilis*, which he supposed occupied a half-way position between *L. bifidus* and *L. acidophilus*.

There was considerable controversy between Tissier and Moro as to the predominant organism in the feces of breast-fed babies, which was finally settled by Moro's admission that *L. bifidus* occurred in far greater numbers. However, both investigators concluded that *L. acidophilus* became predominant in the feces of babies whose chief food was cow's milk.

Rettger and his associates (1912, 1914 and 1921) have shown that the feeding of milk in large amounts, or of lactose, to white rats and human subjects brings about a transformation of the intestinal flora to one in which the lactobacilli of the acidophilus and bifidus type predominate.

Howe and Hatch (1917) in a study of dental caries noted the presence of large numbers of organisms similar to *L. acidophilus* and *L. bifidus*. Boas and Oppler (1895) isolated a species from cases of carcinoma of the stomach which was similar to the lactobacilli in general characteristics. Galt and Iles (1914) observed this organism in three cases of gastric carcinoma and concluded that it was identical with *L. bulgaricus*.

Döderlein (1892) discovered an "acid bacillus" in normal vaginal secretions. Jötten (1922) in an exhaustive comparative study of this organism and *L. acidophilus* concluded that the two species are identical.

EXPERIMENTAL

The present study does not include all known species of the Lactobacillus genus. It deals chiefly with varying strains of what have been designated as *Lactobacillus bulgaricus* (Massol) and *Lactobacillus acidophilus* (Moro) and which have been used in the preparation of sour milk for therapeutic purposes.

A few supposedly closely related forms have been included for the sake of comparison.

The experimental work naturally falls into the five following divisions:

1. Suitable media for use in the studies contemplated.
2. Methods of isolation, and a brief history of the strains of each species exployed, in so far as this was possible.
3. A detailed study of the morphological and cultural characteristics.
4. Determination of fermentative action in milk and various carbohydrates, etc.; and observations of some of the physiological actions of both species upon gelatin and upon milk proteins.
5. An attempted study of the antigenic properties of a few members of both species and complement fixation and agglutination studies with the sera of rabbits immunized by repeated intravenous injections of cultures.

It was hoped that through these experiments new and definite correlations might be established which would enable us more readily to differentiate between *L. bulgaricus* and *L. acidophilus*.

CHOICE AND DEVELOPMENT OF MEDIA

In the isolation and in the study of the cultural and biological characteristics of the *L. acidophilus* and *L. bulgaricus* species, much stress has been placed by various investigators on the medium used. Neither of these types gives a profuse growth in ordinary laboratory media, and some members of the *L. bulgaricus* group will not grow at all in such media. In studies of their action on carbohydrates and other fermentable substances, this has proved to be a very serious obstacle and, no doubt, is responsible for some of the contradictory results reported.

Investigators have employed a diversity of media, generally of special composition. The majority of these special media gave excellent results in so far as growth of the organism was concerned. However, many of the media were such that the carbohydrate constituents could not be controled. In addition, the method of preparation in some instances was rather

laborious and complicated. An attempt has therefore been made to develop a medium with complete control of the carbohydrate content and one which can be simply and easily prepared and yet give consistently good growths of both groups of organisms.

It was shown in a previous report (1922) that nutrient agar made with Difco pepton plus galactose produced a medium which gave sufficient growth of *L. acidophilus* for isolation work and cultural study. However, in the present work it was found that the majority of the strains of *L. bulgaricus* studied would not grow satisfactorily in this medium and that the fault lay not in the carbohydrate but in the pepton.

Opsine, a biuret-free protein digestion product of French manufacture, employed successfully by Robinson and Rettger (1916) was substituted for Difco pepton. An Opsine-meat-extract-agar containing 1 per cent galactose gave consistently good growths of both species of bacteria It was, however, impossible to secure a new supply of opsine as its manufacture had been discontinued, so another medium had to be developed.

Both species grew well in milk, especially *L. bulgaricus;* hence it was thought that a medium containing the nitrogen fractions of milk should furnish the proper food. Gorini (1914) emphasized the need of using a medium made from milk or milk constituents in any study of lactic acid bacteria. Ayers and Mudge (1920) advocated the use of milk powder agar in the determination of bacteria in milk. As a permanently transparent, carbohydrate-free basic medium was desired, it was apparent that one made from the digested proteins of milk should prove more or less satisfactory.

Cole and Onslow (1916) had devised a method for the preparation of a casein digest which in their hands proved to be a very superior medium for bacterial growth. Their method calls for a digestion period of fifteen days, which for practical purposes makes the method rather slow and cumbersome. A modification of this method was adopted.

In the first trials a mixture of 9 parts of casein and 1 part of egg albumen dissolved in 1 per cent sodium carbonate was

digested with fresh trypsin powder for forty-eight hours at 37°C. The resultant digest, when incorporated in a medium with galactose and meat extract, gave excellent growth of *L. acidophilus* and *L. bulgaricus*.

Further trials showed that the egg albumen could be omitted. After a number of experiments involving comparisons with other media, the following method was adopted as "standard" for the preparation of casein digest which was to be employed as the nitrogenous base of all media used in the comparative study of *L. acidophilus* and *L. bulgaricus* in this research.

Two hundred grams of powdered or finely granulated casein are dissolved in 2 liters of 1 per cent sodium carbonate in the following manner. The sodium carbonate solution is heated to boiling and the casein added, a small quantity at a time, with stirring after each addition until completely dissolved. This solution is then cooled to about 40°C. and a water solution (10 to 12 cc.) containing 3 grams of *fresh* Fairchild's trypsin added. After mixing well and adding 25 cc. of chloroform as a preservative, the mixture is introduced into a flask and placed in the 37°C. incubator where it is kept for forty-eight hours. At the end of the forty-eight hours incubation period the mixture is made neutral to litmus with HCl, heated in a double boiler to drive off all chloroform, and then filtered. When chemically pure casein is used the resultant filtrate is clear and has a light amber color. Commercial casein, because of its small lactose content, makes a darker colored product. The insoluble residue does not exceed over 5 per cent of the original casein and is usually considerably less.

In this investigation, a quantity of digest sufficient for several months use was made up at one time. Quantities equivalent to 10 grams of the original casein were placed in cotton-stoppered bottles and sterilized by autoclaving. The contents of each bottle were made up to 1000 cc. with water for each liter of medium desired.

It was determined that for routine use, a tryptic digest of Klim (powered skim milk) could be substituted for casein di-

gest. This gave even slightly better growth of *L. acidophilus* and *L. bulgaricus* than did casein digest media.

In the preparation of Klim digest, it has been found expedient to dissolve the Klim in *cold* sodium carbonate. The quantities employed are the same. It is difficult to secure a clear filtrate after digestion; therefore, the Klim digest was put up in 100 cc. amounts without filtering. During the process of media making, the fine precipitate in the digest was eliminated and the completed medium was practically clear.

The formula for the preparation of media from casein or Klim digest is as follows:

Digest solution corresponding to 10 grams of casein or Klim
3 grams of commercial meat extract
Agar to make the desired consistency
1 per cent carbohydrate (this amount can be cut in half when Klim is used)
Water to make 1000 cc.

No exhaustive chemical analyses of casein or Klim digests as prepared by the methods given, have been made. Qualitative tests have shown the presence of considerable free tryptophane in forty-eight hour digests. Viale (1922) has shown the presence of free tryptophane and cystine in milk, and it may be that these two amino acids, secured by the digestion of the casein, furnish the proper nitrogenous substance for speeding up the growth of *L. acidophilus* and *L. bulgaricus*. Koser and Rettger (1919) showed that tryptophane was the best single amino acid in combination with a synthetic salt mixture for certain bacterial growths. Broth made with a twenty-four hour casein digest, when incubated with *Bact. coli* and incubated for twenty-four hours gave a very intense color reaction for indol with Ehrlich's aldehyde reagent. Commercial pepton broth when employed under the same conditions, gave only a slight color after the same period of incubation. Davis (1917) states that the extent of indol production is a very good practical test for the biologic availability of a pepton.

In the study of the fermentative action of *L. acidophilus* and *L. bulgaricus*, the dilute agar medium devised by Hitchens

(1922) was used in place of broth. At least twice as much growth was obtained in the agar as in broth.

The results of media study in the present investigation may be summarized as follows:

1. Casein and Klim digest media appear superior to ordinary media for the cultivation of *L. acidophilus* and *L. bulgaricus*.

2. Galactose is apparently the most favorable carbohydrate for the development of both species. This supports earlier observations (Kulp and Rettger, 1922).

3. Hitchen's dilute agar is more suitable for obtaining abundant growth of these two organisms than broth.

Tryptic digest of casein or of milk powder (Klim), prepared as herein described, was employed as the nitrogenous base for all media used in this investigation, unless specifically noted otherwise.

METHOD OF ISOLATION

Beijerinck (1889) secured isolations of *L. caucasicus* by employing whey gelatin, made slightly acid with lactic acid. Colonies were picked off and transferred to sterile whey. Johannesen (1897) was the first investigator to use acid broth for the separation of acid-forming bacteria. Heymanns (1898) developed his glucose acetic acid broth for this purpose.

Veillon and Zuber (1898) grew their cultures in deep agar tubes by a modified method of Liborius. The colonies were withdrawn from the lower part of the tube with a sterile pipette or a platinum loop. Tissier isolated *L. bifidus* with the aid of the Veillon tube, while Moro employed acid beer wort broth and agar.

Rettger and his associates have shown that by feeding liberal amounts of dextrin and of lactose to white rats and human subjects the aciduric type of intestinal organisms can be made to develop in the intestines to the almost total exclusion of all other cultivable forms. Advantage was taken of this method to procure isolated strains of *L. acidophilus*. In certain instances where there had been no preliminary carbohydrate feeding, Heymanns' glucose acetic acid broth was employed

as an enrichment medium before dilution plates were prepared from the feces.

After incubation for forty-eight hours at 37°C., typical acidophilus colonies were removed by cutting out a block of agar immediately surrounding the colonies with the aid of a sterile platinum spatula, and transferred to a tube of sterile litmus milk. The agar and the enclosed colony were thoroughly broken up by crushing against the inner wall of the tube. This culture was then incubated until the milk showed an acid curd. To insure purity, this milk culture was plated again and re-isolation made. Usually this procedure was repeated four or five times. Gram stains were made as an additional check upon the purity of the culture.

All strains of *L. acidophilus* employed in this investigation were isolated from typical acidophilus colonies. All *L. bulgaricus* strains were received as pure cultures. These, however, were subjected to re-isolation tests to insure freedom from other forms.

The following is a list of all strains of both species employed in this investigation, with a short history of each.

Sources of bacterial strains employed

L. acidophilus strains R-1-1, S-1, R-J, B-A-3, R-1-1 F and S-d were isolated from the feces of white rats and human subjects. They soon acquired the property of curdling milk.

R-1-5, R-4-8, R-11, 12 X, 13, P, D, Rb-A, Rb-B and Rb-C were strains of *L. acidophilus* isolated from various sources: rats, humans, rabbits and dog—which had all the characteristics of the first group except that they curdled milk slowly and therefore could not be used in the practical production of acidophilus milk. L-43 was an organism secured from the Army Medical School and had been isolated from dental caries.

L. bulgaricus strains B-1, B-3, B-4, B-12 and B-13 were secured from the Dairy Division of the Bureau of Animal Industry. B-12 had been originally isolated by Dr. Lind of Copenhagen from Bulgaricus yogurt. B-13 was the organism which Freudenreich called *B. casei* (epsilon).

Strains F and H were secured from commercial firms which stated

that they were being used exclusively for the production of Bulgaricus milk and tablets.

MORPHOLOGY AND CULTURAL CHARACTERISTICS

Beijerinck (1889) stated that *L. caucasicus* is a non-motile, non-sporing facultative rod-shaped bacterium. The colonies were slow to develop, and at best remained very small. The optimum temperature was 45°C. Freudenreich (1897) reported that *L. caucasicus* grew very slowly at 22°C., faster at 35°C., producing small colonies with hair-like projections around the border. It did not grow upon potato. He described it as a Gram-positive rod with rounded ends, 1μ by 5–6μ, non-motile and without spores. Old cultures showed granules when stained. The organism was killed by exposure to 55°C. for five minutes and when dried, died off within two days at ordinary temperature.

Grigoroff (1905) described his Bacillus A (*L. bulgaricus*) as a long rod, non-motile, growing singly or in chains, and producing no spores. This organism stained well with all basic aniline dyes and was Gram-positive. It was a facultative anaerobe with an optimum temperature of 37°C. In a twenty-four hour broth culture there was turbidity which cleared up within a few days, leaving a heavy sediment. No growth was observed upon potato or in gelatin. Streptobacillus A of this investigator was a Gram-positive rod, growing in chains consisting of from 4 to 20 members. It appeared to be more heat-resistant than Bacillus A, withstanding a temperature of 60°C. for five hours, whereas Bacillus A was destroyed in one hour.

White and Avery (1910) placed the organisms which they studied in two groups, A and B. Those of group A revealed uniformly stained protoplasm when treated with Loeffler's methylene blue or Neisser's stain. Group B showed intensely staining granules. There was more abundant growth of B than A in gelatin, with no liquefaction in either case. In other respects, the organisms of both groups had the same general characteristics of the *L. caucasicus* group.

Moro (1900) described the "Blaubacillus" (*L. acidophilus*) as a non-motile, non-spore-forming rod, 0.6 to 0.9μ by 1.5 to 2μ. It was a facultative anaerobe, growing best at 37°C. Young cultures were Gram-positive, the bacilli becoming negative in old cultures. Morphology also changed in old cultures to a longer and more slender type with the disappearance of threads and the occurrence of degenerating forms. There was scanty growth on slant agar and no growth in gelatin or upon potato. In broth, there was marked turbidity at the end of twenty-four hours, the liquid clearing up quickly after that time, leaving a fine flaky sediment. Two kinds of colonies were observed in agar plates, one like a grain of sand and another with fine threads extending out from a solid center.

Finkelstein's (1900) organisms were quite similar to Moro's except that the former investigator noted occasional branching. Cahn (1901) did not find any branching forms, but in old cultures he observed marked involution forms. He noted a third type of colony formation on agar, namely, a small disc-like colony. Mereshkowsky (1905) labeled Moro's sand-grain colony type 1 and the filamentous colony type 2. Rodella's (1901) description of "Säuereliebender Bacillus im Säuglingsstuhle" agreed with Finkelstein's report.

Tissier (1900) described *L. bifidus* as a strict anaerobe and as being slender, from 2 to 6μ in length, non-motile and non-sporing. This organism appeared very often as a bifurcated (y-shaped) rod. It grew best at 37°C. and was not as strongly Gram-positive as *L. acidophilus*. A temperature of 60°C. killed this species very quickly. Under the microscope agar colonies appeared disc-shaped, with smooth edges and 2 to 3 mm. in diameter. Tissier's *B. exilis* was a small straight uniformly staining rod. It was Gram-positive, grew at 20°C. though the optimum temperature was 37°C. Aerobic colonies were very small and delicate, showing smooth edges.

Jacobson (1908) stated that *L. bifidus* could grow under aerobic conditions in symbiosis. This author noted two types of *L. acidophilus* colonies and three types of organisms: the first type was a large bacillus with square ends growing usually in long

chains; the second was a thin bacillus with round ends and producing no chains; and the third was a very short bacillus. He reported the Gram-staining property as being very variable and stated that no branching forms were observed.

Howe and Hatch (1917) described *L. bifidus* isolated by them as very pleomorphic. On the other hand they noted no marked pleomorphism in *L. acidophilus*. They stated that the size and arrangement of the latter was governed by the medium used.

Rettger and Horton (1914) used the term "X" type for the filamentous colonies of *L. acidophilus*, and "Y" type for the sand grain-like colonies. These investigators suggested a close relationship between the "Y" type and *B. exilis* of Tissier. Rahe (1914) found both "X" and "Y" types in his isolations of aciduric bacteria from the intestines. Sandberg (1904) had reported "X" and "Y" types of colony formed by the Boas-Oppler bacillus.

Jötten (1922) found the morphological and cultural characteristics of Döderlein's "Vaginalbacillus" to be identical with those of *L. acidophilus*.

MORPHOLOGY

All strains of the species employed in this study were Gram-positive rods with rounded ends. No motility was observed in hanging drop preparations taken from twenty-four hour sugar broth cultures, and there was no evidence of spore formation. When stained with Neisser's stain or Loeffler's methylene blue, some organisms stained evenly, while others showed granules. However, these characteristics did not appear to be stable, nor could they be used to differentiate species. Granule formation appeared to a greater extent in the older than in young cultures. No branched forms were seen. The orientation generally showed considerable variation, even in the same culture. In young cultures the width of the rod was quite constant. In old cultures, almost all shapes and sizes could frequently be observed. Generally the majority of organisms in old cultures became Gram-negative.

TABLE 1

	TWENTY-FOUR HOUR SUGAR BROTH CULTURES	FORTY-EIGHT HOUR SUGAR AGAR COLONIES	TWENTY-FOUR HOUR AGAR SLANT	YOUNG MILK CULTURES	OLD MILK CULTURES
B-1	Pairs + 0.8 x 1.5–2	Pairs + 0.8 x 2–4	Sh. ch. + 0.6 x 2–8	Sh. ch. + 0.6 x 2–5	No. ch. – 0.8 x 2–4
B-3	Lo. ch; filam. + 0.5 x 2–6	No. ch. + 1.0 x 2–6	No. ch. + 0.8 x 3–6	Sh. ch. + 0.6 x 4	No. ch. – 0.5 x 2–4
B-4	Sh. ch. + 0.6–0.8 x 3–8	No ch. + 1.3 x 3–8	No ch. + 0.8 x 3–10	ch. + 0.7 x 4–8	No ch. – 0.6 x 3–6
B-13	Lo. ch. + 0.6 x 3–10	Sh. ch. + 0.5 x 2–4	Ch.; rds. + 0.6–8 x 2–6	Ch. + 0.6–1 x 4–6	No ch. + & – 0.6 x 4–10
F	Sh. ch. + 0.8–1 x 3–8	No ch. + 1.0 x 6–8	Few ch. + 0.8 x 4–8	V. lo. ch.; filam. + 0.6–0.8 wide	Lo. ch.; filam. – 0.4 wide
H	Few ch. + 0.8 x 4–8	Few ch. + 0.6 x 3–8	Few ch. + 0.8–1 x 3–6	Few ch. + 0.6 x 3–8	No ch. – 0.5 x 2–8
R-1-1	No ch. + 0.6 x 1–2	No ch. + 1 x 2–4	Sh. ch. + 0.7 x 1.5–3	Lo. ch.; filam. + 0.5 wide	Slender rods – Gran.
S-1-3	Lo. ch. + 0.6 x 1.5	Few ch. + 1.0 x 2–6	Chains; filam. + 0.6 x 2–8	Lo. ch.; filam. + 0.6 wide	No ch. + & – 0.6 x 3–4
S-d	No ch. + 0.7 x 3–4	Lo. ch.; filam. – 0.7–1 x 2–6	Sh. ch. + 0.8–1 x 2–6	F. sh. ch. + 0.8 x 3–6	Sh. ch.; granular – 0.4 x 2–3

TABLE 1—*Continued*

	TWENTY-FOUR HOUR SUGAR BROTH CULTURES	FORTY-EIGHT HOUR SUGAR AGAR COLONIES	TWENTY-FOUR HOUR AGAR SLANT	YOUNG MILK CULTURES	OLD MILK CULTURES
Dog	F. sh. ch. + 0.5 x 1.5–2	No ch. + 0.8 x 2–4	F. sh. ch. + 7 x 1.5–4	Chains + 0.5 x 4	No ch. + & – 0.3 wide
R B	Pairs + 0.6 x 1–3	Pairs + 0.6 x 1.5–3	Sh. ch. + 0.6 x 1.5–3	Sh. ch. + 0.6 x 4–8	Sh. ch. + & – 0.5 x 4–8
R-1-5	Sh. ch. + 0.6 x 1.5–4	Sh. ch. + 0.6 x 1.5–3	Sh. ch. + 0.8 x 3–4	Chains + 0.6 wide	Chains + & – 0.6 x 3–4

The figures refer to the size in microns of individual bacteria.

+ and – mean Gram-positive and Gram-negative.

Ch. = chains; sh. = short; lo. = long; filam. = filaments; f. = few; v. = very; rds. = rods.

Gram stains of several members of each species were made from twenty-four hour broth cultures, agar colonies, agar slant growths and young and old litmus milk cultures. The size, orientation, and Gram-staining properties are given in table 1.

It is very evident that the morphological differences between these species are very slight and cannot be used as a basis for separation. The orientation and size of the various strains studied appear to be influenced to a large extent by the medium in which they are cultivated.

CULTURAL CHARACTERISTICS

The cultural characteristics of all organisms employed in this study were determined in sugar agar plates, potato, litmus milk, whey and in carbohydrate broth and gelatin. All cultures were incubated at 37°C. except where otherwise noted.

Potato. None of the strains of the two species grew on this medium.

Broth. Growth in whey-pepton and in sugar broths had the same general characteristics. The medium became turbid within twenty-four to forty-eight hours. On longer stand-

ing the liquid became clear. Some growth stuck to the walls of the test tubes; the remainder formed a sediment, either viscous or granular.

Litmus milk. This medium was curdled at 37°C. by all strains studied. The period of incubation before curdling occurred varied from twenty-four hours to several days.

The curd produced by *L. bulgaricus* was firm, with no extrusion of whey. *L. acidophilus* produced a softer curd, with slight extrusion of whey. The action by both organisms upon litmus was very much like that of *Streptococcus lacticus;* that is, reduction occurred with subsequent oxidation.

At room temperature (20° to 25°) growth, when evident, was very slow. B-3 curdled the milk in twelve days, B-4 and B-13 in thirty days; no other strain produced a curd after thirty days, and only a few caused a reddening of the litmus.

Slant Agar Growth. The cultures of both species showed practically the same characteristics. The agar was inoculated by drawing a loopful of a twenty-four hour broth culture over the surface. Growth was recorded after twenty-four hours incubation at 37°C. The growth was not heavy in any instance. B-13 and F gave a thin granular growth; while those of B-3, B-4 and B-12 were barely visible and appeared spicule-like. The growth of the remaining strains employed can best be described as being delicate and streptococcus-like to the naked eye or when slightly magnified.

Gelatin Stab Cultures. These were made from twenty-four hour slant agar cultures. The gelatin medium was incubated at 20°C. B-4, B-12 and S-1-3 did not reveal any growth in fifteen days, B-3, B-13, F, R-11 and R-1-1 showed development along the stab, with hair-like lateral branches. The growths of R-1-5, R B, B-1, P and B-A-3 were filiform. There was little or no development upon the surface of the medium and no evidence of liquefaction.

Agar Colonies. Characteristic colonies were formed by all of the *L. bulgaricus* strains but B-1. All colonies showed comparatively long interlacing and hair-like filaments radiating outward from a common center, best described as an extreme

"X" type. B-1 at times produced a solid colony with an even edge.

The different strains of *L. acidophilus* formed two main types of colonies, the almost smooth sand grain or streptococcus-like "Y" type and the filamentous "X" type; all graduations between these two were present. At times there also appeared small disc-like colonies. Surface colonies between the agar and the bottom of the Petri dish are often very filamentous and spreading. The variation in colony formation is a source of annoyance to anyone who is working with *L. acidophilus*, as the appearance of the "Y" type or disc-like colonies causes suspicion as to the purity of cultures. However, the colony type is not necessarily stable and re-isolations from any one type will at times produce colonies of either of the other two types. All primary isolations of the species labled *L. acidophilus* in this investigation were made from extreme "X" type colonies; yet the other two forms have appeared quite often in subsequent platings.

What the exact conditions are that determine the type of colony have not been discovered in this investigation. The "X" type appears more often in crowded plates, suggesting that metabolic products may influence the colony formation. This point, however, has not been confirmed.

PHYSIOLOGICAL ACTION

Fermentation studies. In the comparative study of members of the Lactobacillus genus, considerable weight has been given by many investigators to the fermentative action of these organisms in milk and other fermentable substances. Some workers have emphasized the ability to ferment definite substances as a basis of differentiation.

Grigoroff (1905) observed the necessity of sugar media for cultivation of his Bacillus A (*L. bulgaricus*) and Streptobacillus C. Bacillus A produced large quantities of lactic acid in milk, curdling it at a temperature of 42°C. in about five hours. This organism also attacked maltose, levulose and sucrose. It did not ferment rhamnose, dulcitol and sorbitol. The streptobacillus C curdled milk and attacked sucrose without inversion;

also glycerol and levulose. It had no action upon mannitol, maltose, rhamnose, dulcitol and sorbitol. From whey fermented by this organism, he secured a substance which gave the iodoform test for alcohol. Grigoroff makes no mention of gas formation.

Bertrand and Weisweiller (1906) reported that *L. bulgaricus* produced from 2.5 to 3 per cent lactic acid in milk, most of which was dextrorotatory. The organism also produced small amounts of succinic and formic acids in the same medium. These investigators claimed that this species acted by means of an intracellular lactase.

Cohendy (1906) isolated an organism from Bulgaricus milk which developed an acidity of 3.2 per cent as lactic acid in milk cultures incubated at 36°C. It also fermented maltose, sucrose levulose and glucose, and required carbohydrate for growth in any medium.

Bertrand and Duchacêk (1909) made an exhaustive study of the fermentative action of *L. bulgaricus* upon various carbohydrates and higher alcohols. They found that this organism produced inactive lactic acid with small amounts of formic, acetic and succinic acids from lactose, galactose, glucose, levulose and mannose. Levulose and mannose were less easily fermented than the others. Sucrose, maltose, the pentoses, sorbitol and mannitol were not acted upon. In milk the action of this organism was about the same except that the greater amount of the lactic acid was the dextrorotatory form.

Rahe (1914) made a thorough study of a number of species of the lactobacillus genus which he divided into three groups according to their action in milk and in a medium containing maltose.

Variety A clotted milk and produced no acid from maltose. Variety B clotted milk and produced acid from maltose. Variety C did not clot milk in six days and produced acid from maltose.

All strains of variety A were secured from type cultures of *L. bulgaricus*, fermented milks and bulgaricus tablets. There were 12 strains of this variety and it is of interest to note that

7 of these were reported as producing no acid in a levulose medium; 9 did not attack sucrose.

Strains of Variety B, 21 in number, were isolated from intestinal sources, with 2 exceptions. One of these was isolated from saliva and 1 from fermented milk. All of these fermented levulose, and all but 1, sucrose.

Variety C, 20 strains, with the exception of 2 from saliva and 1 from a tablet, were secured from intestinal sources. All of these strains fermented maltose and levulose, and all but 1, sucrose. Raffinose and mannitol were irregularly fermented by varieties B and C, while variety A did not attack these two substances at all. Variety A did not grow in sugar-free media; varieties B and C showed slight growth after seventy-two hours.

Moro (1900) stated that his original *L. acidophilus* produced acid in milk, curdling it in from three days to three weeks when incubated at 37°C. It developed considerable amounts of acid in carbohydrate media, from glucose and sucrose more readily than from lactose. No gas was formed by the organism in any medium studied. Kendall (1910) in a study of lactobacilli from the intestines corroborated Moro's conclusions.

Howe and Hatch (1917) reported that members of the Moro-Tissier group found in dental caries fermented, sucrose, glucose, lactose, maltose and raffinose with production of acid, but no gas.

Jötten (1922) observed that *L. acidophilus* and Döderlein's "Vaginalbacillus" fermented glucose, levulose, lactose, sucrose, maltose and mannitol. The pentoses and higher alcohols were not attacked. Glucose was fermented by a few strains of each species and glycogen by all strains of both. In milk culture incubated at 37°C. for fifteen days, the acidity ranged from 0.34 to 1.4 per cent, calculated as lactic acid.

Heineman and Ecker (1916) found that 3 strains of lactobacilli from gastric ulcers, which they called Boas-Oppler bacilli did not ferment maltose and produced 1.57 per cent levorotatory lactic acid in milk in from four to six days at 37°C.

The basic medium adopted for the fermentation studies in this investigation was a casein digest broth plus 0.1 to 0.15

per cent agar. All fermentable test substances were sterilized by filtration, to avoid decomposition of the carbohydrates. Five or 10 grams of test substance were dissolved in 100 cc. of distilled water. This solution was filtered through a sterilized Berkefeld candle into a sterile filtering flask. One-half cubic centimeter of this filtrate was added to each 5 cc. of sterile basic medium with a sterile pipette, making approximately a 0.5 or 1 per cent concentration of test substance. The medium prepared in this manner was always tested for sterility by incubation at 37°C. and at room temperature for at least two days before use.

Fresh skimmed milk, sterilized by autoclaving, was employed in the study of the action of *L. acidophilus* and *L. bulgaricus* on milk. Heavy inoculations are necessary in a study of these organisms, in order to secure good growth in a reasonably short period of time. In fact any thing short of the pipette method is inadequate. This applies not only to milk, but to all other media as well. The following method was used for supplying concentrated viable inoculum in small amounts without accompanying carbohydrates, etc. Young, vigorously growing broth cultures were centrifuged. After the removal of the supernatant liquid, the well-packed organisms were suspended in a sufficient amount of physiological saline solution to give the suspension a turbidity of about 6 on the McFarland nephelometer scale (1907); 0.1 cc. of this suspension was employed as the inoculum in all cases.

In order to determine to what extent the various test substances were broken down by heat during sterilization, control tubes of autoclave-sterilized casein-digest dilute agar containing the test substances were carried along with the adopted test medium in which the test substances had been sterilized by filtration. This brought out a very interesting point which will be discussed later.

The action of the species studied upon the various test substances in dilute agar was measured by the drop in the pH as determined by the colorimetric method of Clark and Lubs (1917). In milk, the acidity was determined by titration.

Preliminary experiments showed that 37°C. was the optimum temperature for all species used in this investigation. Therefore, the various casein digest dilute agar media were incubated at this temperature for seventy-two hours and the extent of growth and drop in the pH noted.

All determinations were carried out in duplicate or triplicate. A tube of uninoculated test medium as well as an inoculated tube of the basic medium were run with each strain studied. The purity and viability of each culture used for inoculum was tested by plating 0.1 cc. in galactose casein digest agar.

The following test substances of the highest purity were employed:

Hexoses
Glucose
Levulose
Galactose
Mannose

Disaccharides
Lactose
Maltose
Sucrose
Trehalose

Trisaccharides
Raffinose
Melezitose

Pentoses
Arabinose
Xylose

Polysaccharides
Dextrin

Alcohols
Glycerol
Mannitol
Dulcitol
Sorbitol
Rhamnose

Glucosides
Inosite

The results of several experiments conducted over a period of a year are summed up as follows:

ACTION IN VARIOUS CARBOHYDRATE DILUTE AGAR MEDIA

1. Dextrin was at best but slightly affected by the various strains; the results were variable.

2. Mannitol, arabinose, glycerol, dulcitol, inosite, rhamnose, xylose, sorbitol and melezitose were apparently not attacked by any of the strains.

3. All strains studied fermented levulose media with acid production when such media had been sterilized by heating. There were striking differences, however, when Berkefeld filtered

levulose was used. None of the *L. bulgaricus* strains fermented this medium when the pure unheated sugar was employed. Two samples of levulose which were not of highest purity were fermented by B-1 and F, even when these particular samples had been sterilized by filtration.

The fermentative action of some of the *L. bulgaricus* strains upon heated levulose was not as vigorous as upon other fermentable sugars; nevertheless, in practically every experiment in which heated levulose was employed there was an appreciable growth with a corresponding drop in the pH of the test medium. All the strains of *L. acidophilus* but two fermented both heated and unheated levulose media. The two exceptions were very slow growers, and this may in part at least account for their indifference.

4. Acid production in maltose media, which has been suggested by Rahe and others as an important differential test for the separation of the two species was somewhat disappointing from this standpoint. B-1 and B-13 attacked it vigorously while all other strains of *L. bulgaricus* failed entirely to act upon it. This is in agreement with previous experiences in this laboratory. These may be borderline types, especially B-1 which attacked sucrose. All strains of *L. acidophilus* employed fermented the maltose.

5. B-1 was the only *L. bulgaricus* species to produce acid from sucrose, while all strains of *L. acidophilus* gave positive acid production.

6. Lactose, glucose and galatose were broken down with production of acid by all members of the two species.

7. Trehalose showed the same variation with *L. bulgaricus;* as did maltose; *L. acidophilus* also was variable in attacking this sugar.

8. *L. bulgaricus* gave negative results in the raffinose medium; while *L. acidophilus* appeared quite variable in its action upon this sugar.

9. When there was acid production in any medium, the hydrogen-ion concentration varied considerably. However, the lower limit of the drop appeared to lie at about pH 3.6 to 4.0 for

all strains. This is in agreement with the findings of Clark (1916).

10. Glucose, galactose and lactose media in fermentation tubes were employed in an attempt to demonstrate gas production by members of these two species. There was complete absence of gas in all cases after twenty-four, forty-eight and seventy-two hour periods of incubation at 37°C.

11. With the exception of levulose and possibly mannose, sterilization of media containing the various fermentable substances by autoclaving at 15 pounds extra pressure for fifteen minutes did not appear to break down the test substances sufficiently to produce appreciable change in hydrogen-ion concentration when inoculated with members of the species that do not attack these sugars normally.

The study of the fermentative activity of the various strains of *L. acidophilus* and *L. bulgaricus* when grown in ordinary litmus milk gave the following results:

1. The milk generally curdled when the reaction reached about 0.5 per cent acid calculated as lactic acid by the titration method.

2. Acidity increased faster at 42° than at 37°C. but after a period of thirty days incubation the greatest acidity was obtained at the latter temperature.

3. All *L. bulgaricus* strains curdled milk within twenty-four hours at either 37° or 42°C.

The time required for curdling by *L. acidophilus* strains varied from forty-eight hours to ten days.

4. The acidity produced by different strains of *L. acidophilus* at 37°C. varied from 0.86 to 2.39 per cent; at 42°C. this acidity varied from 0.72 to 1.98 per cent.

5. *L. bulgaricus* strains developed at 37°C. from 1.82 to 3.15 per cent acidity; at 42°C. from 1.82 to 2.52 per cent acidity.

6. There was no formation of gas in milk by any strains employed in this investigation.

7. A distillate from whey broth cultures which had been incubated at 37°C. for twelve days gave an iodoform test for alcohol.

ACTION UPON MILK PROTEINS AND GELATIN; INDOL FORMATION

Grigoroff's (1905) Bacillus "A" did not produce indol and a casease could not be demonstrated. Cohendy (1906) stated that the species which he isolated did not visibly attack the casein in milk and had no action upon fibrin, egg white or sugar gelatin. Bertrand and Weisweiller (1906) reported that *L. bulgaricus* digested approximately one-tenth of the casein in ordinary milk culture.

Supplee (1917) noted that *L. bulgaricus* in a milk culture brought about a decrease in the casein and albumin nitrogen; there was a corresponding increase in pepton, diamino and ammonia nitrogen.

Tissier (1905) stated that *L. bifidus* attacked only proteoses, with liberation of ammonia. No indol was formed.

Kendall (1910) reported that *L. acidophilus* did not induce proteolysis and did not form indol from trytophane. Howe and Hatch (1917) in their study of the acidophilus-like organisms of dental caries, reported that these forms did not produce ammonia or indol in pepton media.

Because of the fact that normal members of these two species (*L. acidophilus* and *L. bulgaricus*) will not grow to any appreciable extent in sugar-free media it was necessary in this investigation to study their action upon gelatin and milk proteins in the presence of available sugars.

Ten per cent gelatin medium was made up with casein digest and 0.1 per cent galactose added before sterilization. This small amount of sugar had been determined as being sufficient for considerable growth of these organisms.

Skimmed milk was employed as the medium for observation of the action upon milk proteins.

Tubes of the sugar gelatin were inoculated with 0.1 cc. of a vigorous broth culture of the organism. Sufficient sterile lactic acid to make 3 per cent acidity was added to two tubes to be used as controls. All tubes were sealed by saturating the cotton plug with melted paraffin and then incubated at 37°C. for over a month.

The cultures were tested for liquefaction by removing to a cold place every four days and noting whether the medium became solid. No softening of the gelatin was induced by any of the strains employed after thirty days incubation. All cultures showed very good growth.

For the purpose of studying the action of both species upon the proteins of milk, the following procedure was adopted. Exactly 10 cc. quantities of skimmed milk were sterilized in cotton-plugged test tubes. Some of these were inoculated with 0.1 cc. broth cultures of F, H, B-1, Dog, R-1-1 and R-1-5. Varying amounts of sterile lactic acid were added to other tubes of milk so as to make the acid concentration in these tubes equal to 1, 2, 3, and 4 per cent. These acidified tubes and uninoculated, non-acidulated milk were taken as controls. All tubes were sealed with melted paraffin and placed at 37°C. where they were kept for three weeks.

At the end of this period the following nitrogen determinations were made. The entire contents of each tube were used for each determination so that no correction had to be made for evaporation. All determinations were made in duplicate.

1. Total nitrogen of the milk in two control tubes was determined by the Kjeldahl method.

2. Total protein nitrogen determinations were made of control tubes of 1, 2, 3 and 4 per cent acid milk and of each milk culture.

Ritthausen's method (Leffman and Beam) was employed. The proteins were precipitated by copper sulphate solution and sodium hydroxide. After filtering by decantation and washing Kjeldahl nitrogen determination was made upon filter paper and precipitate together.

3. Pepton and diamino nitrogen was determined in the filtrate from (2).

The filtrate was made acid with 0.5 cc. of 50 per cent sulphuric acid and 6 cc. of a 15 per cent solution of phosphotungstic acid added. After stirring well, this mixture was allowed to stand over night, so that the precipitate could settle out.

The supernatant liquid was poured upon a filter-paper and finally the precipitate itself was washed upon this paper by means of a wash-bottle containing a 2.5 per cent solution of phosphotungstic acid. After washing with this same solution, paper and precipitate were transferred to a flask and nitrogen determined by the Kjeldahl method.

The results shown in table 2 indicate some breaking down of the milk proteins, due to digestive action, by both *L. acidophilus* and *L. bulgaricus*. The acidified controls showed an

TABLE 2

Showing action of L. acidophilus and L. bulgaricus on milk proteins

	TOTAL NITROGEN	TOTAL PROTEIN NITROGEN		PEPTON DIAMINO NITROGEN		AMINO, AMMONIA, ETC., NITROGEN	
	mgm.	mgm.	per cent T. N.	mgm.	per cent T. N.	mgm.	per cent T. N.
Control	527.1	489.3	92.8	17.5	3.32	20.3	3.85
H	527.1	447.3	84.9	35.0	6.64	44.8	8.50
B-1	527.1	443.1	84.1	37.1	7.04	46.9	8.90
F	527.1	445.7	84.6	28.0	5.31	53.4	10.1
Dog	527.1	455.7	86.5	37.8	7.17	33.6	6.38
R-1-1	527.1	430.5	81.7	55.3	10.5	41.3	7.84
R-1-5	527.1	463.6	88.0	33.6	6.37	29.9	5.67
1 per cent lactic	527.1	480.2	91.1	25.9	4.91	21.0	3.99
2 per cent lactic	527.1	485.8	92.2	23.8	4.51	17.5	3.32
3 per cent lactic	527.1	472.5	89.6	38.5	7.30	16.1	3.06
4 per cent lactic	527.1	470.4	89.2	41.6	7.89	15.1	2.87

T. N. = total nitrogen.

Results are given in milligrams of nitrogen per 100 cc. of milk and in percentage of total nitrogen.

increase in the pepton and diamino nitrogen fraction, the greatest being in the case of the 4 per cent acid milk. With the exception of R-1-1, there was no great difference in this respect between the artificially acidified milk and the inoculated milk. However, taking into consideration that the acidity of the acidophilus milk was not over 2 per cent and that of the bulgaricus milk not over 2.8 per cent, there is some evidence that these organisms have produced more pepton and diamino acids from the milk proteins than is due to acidity alone.

The greatest evidence of digestion by these organisms is in the amounts of residual nitrogen, after the total protein and pepton-diamino fractions have been determined. The acidified milk gave practically the same amount as the control, while there was a considerable increase in the inoculated milk.

A preliminary experiment had shown that there was very little ammonia nitrogen in the inoculated milk; therefore, the greater part of this residual nitrogen was probably non-amino nitrogen. The increase of the inoculated milk over the control milk can be stated as due to a digestion of the milk proteins (presumably casein) by these organisms.

It may be stated that approximately from 2 to 6 per cent of milk proteins is broken down by *L. acidophilus* and *L. bulgaricus*.

Indol production of these species was studied by growing the various strains for five days at 37°C. in 0.1 per cent galactose casein digest broth, and in the same medium plus a trace of added tryptophane. Negative results were secured in every instance.

VIABILITY AND THERMAL DEATH POINTS

The viability of different strains of each species was determined in milk, in Klim digest and in whey pepton broths.

Milk cultures of *L. acidophilus* and *L. bulgaricus* were kept at room temperature and tested for viability by withdrawing 1 cc. and transferring to sterile litmus milk. Growth was indicated by the production of a typical curd. *L. acidophilus* was found to be decidedly viable after three months, while *L. bulgaricus* died off in about six weeks, under the conditions under which the experiment was carried out.

Viability tests on milk cultures kept at 37° and at 42°C. also showed a longer duration of life of *L. acidophilus* than of *L. bulgaricus* under the given conditions. All of the strains of the latter organism failed to revive after fourteen days incubation at both 37° and 42°C., while all of the 15 acidophilus strains grown at 37°C. gave the milk curdling test after eighteen days, and all but 3 were positive at the end of eighteen days incubation at 42°C. Furthermore, none of the *L. bulgari-*

cus strains developed on agar plates when plated out after thirty days incubation at 37° and 42°C., while 9 of the 15 strains of *L. acidophilus* employed gave colonies on the agar plates.

In Klim digest broth cultures kept at refrigerator (8° to 10°C.) and at room temperature there was little difference in the viability of the two species. The *L. bulgaricus* cultures remained viable three, four and five days in the ice box and two, three and four days at room temperature, and *L. acidophilus* three, four and five days and two, four and six days, respectively.

In pepton whey broth the viability of both species was distinctly greater than in Klim digest broth, due in part at least, to the buffering action of the pepton. *L. bulgaricus* remained viable for six, seventeen and forty-two, and three, eight and thirty days, and *L. acidophilus* for six, seventeen, thirty and forty-two, and three, six, seventeen and thirty-five days, respectively, at the ice box and room temperatures.

In ordinary sugar broths viability persists for only a very short time. Milk is preëminently the best medium for keeping the organisms of both species alive. According to Jones (1916) this is due to the buffer action of the milk proteins, which prevent dissociation of H-ions and therefore enable bacteria to grow and continue using carbohydrates.

The resistance of several strains to drying was determined in the following manner. A comparison was made between centrifuged twenty-four hour broth cultures suspended in saline solution and twenty-four hour pepton whey broth cultures. Pieces of sterile filter paper were saturated with very heavy cultures, prepared as noted. These pieces of paper were kept at room temperature and allowed to air-dry. Platings were made by mixing several pieces of paper in agar and incubating at 37° to 40°C. This procedure was repeated at intervals of a day or two until platings finally revealed no growth.

No colonies appeared on any of the plates from paper plated out after two days drying. Desiccation therefore, quickly killed off both species.

The thermal death point of three representative strains of each species was ascertained. According to the results, *L.*

acidophilus has a somewhat higher thermal death point than *L. bulgaricus*, namely 63° to 65°C. as compared to 57° to 61°C. This difference may possibly be explained as being due to a difference in the hydrogen ion concentration of the twenty-four hour cultures preliminary to centrifuging.

SEROLOGICAL RELATIONSHIPS

An exhaustive review of the literature covering the investigations dealing with *L. acidophilus* and *L. bulgaricus* reveals practically nothing in regard to their serological relationships. Jötten (1922) reports that the complement binding properties of *L. acidophilus* and Döderlein's bacillus justify the classification of these forms as one and the same organism.

Jötten immunized rabbits by giving 9 intravenous injections of saline suspension of agar slant cultures. He attempted agglutination studies with the sera obtained, but failed because the antigens agglutinated spontaneously. He was also unable to secure any positive results from the precipitin reactions. In complement fixation studies he obtained complete cross-fixation with sera and antigens of both species. However, he does not give complete details as to the technic which he followed.

In the present investigation an attempt was made to study the serological reactions of the sera of two lots of rabbits, one group of which had been immunized against *L. acidophilus* and the other against *L. bulgaricus* by repeated intravenous injections of cultures of these organisms.

The first experiment on serological relationships was begun with the idea of making an agglutination study. The antigens were secured in the same manner as the antigens for immunization. The organisms were suspended in phenolized saline for preservation. For use in the test, very light suspensions (nephelometer 0.3) were employed. The agglutination tests had to be abandoned, however, owing to spontaneous agglutination of antigens.

Complement fixation tests were conducted with autolyzed antigen which was prepared by suspending centrifuged cells

from sugar broth cultures in saline solution, adding sodium carbonate to give a pH of 9.5, and keeping the alkaline suspension at 37°C. for four days. The extraction of soluble antigen was in all probability due to solution by the carbonate as well as to actual autolysis. The antigens were tested before incubation by Gram staining and found to be made up of even staining, strongly Gram-positive rods. After the four days incubation they were again examined. Practically all of the cells were Gram-negative and some showed complete disruption. Two

Complement fixation results

		F serum diluted 1:25 0.4 cc. used	D serum diluted 1:10 0.4 cc. used	R-1-1 serum diluted 1:20 0.3 cc. used
Bulgaricus	B-1	0	3+	4+
	B-3	4+	3+	4+
	B-4	4+	3+	4+
	B-12	4+	3+	3+
	B-13	4+	2+	4+
	F	4+	3+	3+
	H	4+	3+	2+
Acidophilus	R-1-1	3+	4+	4+
	D	3+	4+	4+
	S-1	3+	3+	4+
	R-1-5	3+	4+	4+
	R B	4+	4+	4+
	B-A-3	0	3+	+
	L-43	0	0	2+

of the antigens (R-1-1 and Dog), were practically clear solutions after incubation: the third showed more or less turbidity.

Four rabbits were immunized by the same method as was used before, with R-1-1, Dog, F, and H strains. Intravenous injections were made at four day intervals until 9 injections had been given. Eight days after the last immunizing dose, the animals were bled and the sera secured as in the former experiment. No preservative was added to these sera.

Serum H did not prove to be strong enough for use. There-

fore three sera were employed in the complement fixation tests. F was bulgaricus immune serum, D and R-1-1 were acidophilus sera. These sera were not anticomplementary in any dilution employed.

The homologous antigen and thirteen heterologous antigens were tested for their complement fixing powers with each serum. Three tenths cc. of antigen was adopted as the antigenic unit. B-A-3 and L-43 were markedly anticomplementary in amounts over 0.6 cc., while none of the others showed anticomplementary action in 1 cc. amounts.

This table shows positive fixation between heterologous antigens and sera in nearly all tests. B-A-3, L-43 and possibly B-1 apparently do not belong to either of these two groups, in so far as serological tests show. If any weight is to be given to cross fixation, this set of experiments indicates a close relationship between the other 11 strains employed (6 of *L. bulgaricus* and 5 of *L. ancidophilus*).

DISCUSSION

The results of this investigation indicate a very close relationship between the members of the *L. acidophilus* and *L. bulgaricus* types studied.

The strains used are fairly representative of the organisms found in fermented milks and in the intestines of man and animals, characterized by the production, in part or altogether, of sea-urchin-like colonies in agar and in gelatin media. The *L. bulgaricus* type appears to be more constant than the *L. acidophilus* type in this respect.

The morphology of the two groups is quite similar.

There are differences in their action upon maltose, sucrose and unheated levulose. Other physiological reactions differ only in degree.

The results of complement fixation tests show cross-fixation between heterologous immune sera and antigens. There is a quantitative difference in some cases, but this is no greater than would be expected between different strains of the same species.

The one outstanding difference between the two types is their habitat. In no case in the course of this investigation has an organism been isolated from feces which had the characteristics of a typical *L. bulgaricus* strain. Herter and Kendall (1909), Rahe (1915) and Rettger and Cheplin (1921) have proved that *L. bulgaricus* cannot be implanted in the intestines of man or animals. The present authors in the course of an unfinished experiment were not able to recover a non-maltose fermenting *L. bulgaricus* from the feces of white rats after two weeks feeding of large milk cultures of *L. bulgaricus* plus lactose.

On the other hand, the ingestion of milk or broth cultures of *L. acidophilus*, or the use of a high lactose or dextrin diet alone, (Rettger and Cheplin) brings about a transformation of the intestinal flora to such an extent that *L. acidophilus* becomes the predominating organism. Therefore, it can be concluded that the normal habitat of *L. acidophilus* is the intestine of man and animals. Other Gram-positive bacteria present are not to be considered as the same species. These can be excluded by means of their colony formation. This will eliminate such types as L-43 and B-A-3 employed in this study.

From the manner in which the organism grows in milk this medium can be considered as giving optimum conditions for the growth of *L. bulgaricus*. However, this does not explain its original source.

The question of the origin of *L. bulgaricus* can only be a matter of conjecture. Was it always a so-called saprophyte, living in milk, possibly coming from soil? Or, is it a degenerate form of *L. acidophilus*, requiring milk as a food because of long acclimatization to this medium? The authors offer the latter as a suggestion for the following reasons.

1. In milk, *L. bulgaricus* has lactose and the hydrolysis products of this sugar, galactose and glucose, to supply its carbohydrate requirements. There is no levulose or maltose in milk. Therefore, because of years of sojourn without these sugars, *L. bulgaricus* may have lost its power to utilize them.

2. As has been stated, *L. bulgaricus* grows best in milk. On the other hand *L. acidophilus*, which in primary isolation grows

poorly in milk, can become acclimated so that it develops very rapidly.

3. *L. acidophilus* grows well in practically all ordinary sugar media. In the intestine it has a great variety of nitrogenous food to choose from and therefore can adapt itself to various nitrogenous constituents in media. *L. bulgaricus* on the other hand developed poorly or not at all in our experiments except where the nitrogenous constituents were derived from milk. Is this fastidiousness not due perhaps to the same cause as its inability to use sugar other than lactose or its immediate derivatives?

Heineman and Hefferan concluded that the whole group of lactobacilli should be placed in one species, *L. bulgaricus*.

From a study of the descriptions given of *B. lebenis*, *Streptobacillus* lebenis, *Bact. mazun*, *Bact. caucasicum*, *B. casei* (epsilon), etc., there can be little doubt that these organisms are identical with *L. bulgaricus* (Massol). The type isolated by various investigators from intestinal contents, which produces the "X" type colony, and probably the "Y" type as well, is without question *L. acidophilus* (Moro.) Furthermore, the Boas-Oppler bacillus and Döderlein's bacillus may be included in this division. It is quite probable that the aciduric silage bacteria, organisms isolated from dental caries, *B. panisfermentati*, etc., are not identical with either *L. bulgaricus* or *L. acidophilus*.

If *Bact. abortum* and *Bact. melitensis*, as well as *Bact. coli* and *Bact. aerogenes* and certain other closely related members of the colon-typhoid group are to be considered as separate species, then *L. acidophilus* and *L. bulgaricus* may also be considered as distinct and separate species.

However, the authors suggest that the Gram-positive rods designated *L. bulgaricus* and *L. acidophilus* be placed in the same species, with *L. acidophilus* as the central type. *L. bulgaricus* may be considered a variant of this type, variation being due to long culturing in milk. The chief differential characteristic of this variant is the fact that it has lost its ability to develop in the intestine. The central type, *L. acidophilus*, is a normal intestinal form which can continue to develop in the intestine, even when supplied as a milk culture.

SUMMARY AND CONCLUSIONS

In the comparative study of *L. acidophilus* and *L. bulgaricus* the following points received chief emphasis.

a. Development of suitable media
b. Source and habitat
c. Morphology and cultural characteristics
d. Physiological action
e. Viability and thermal death points
f. Immunological relationships

The results may be stated briefly as follows:

1. Casein digest media proved to be particularly valuable in the present study of *L. acidophilus* and *L. bulgaricus.*

2. These two species are quite similar in morphology and cultural characteristics. Differences, when observed, were quantitative rather than qualitative.

3. The action upon maltose, sucrose, and levulose appears to furnish a valuable means of separating strains of these two groups, which are now being employed in the production of fermented milks. In other physiological aspects they are very much alike.

4. The viability and thermal death points are somewhat variable for different strains of each species.

5. The immunological studies showed no greater difference between *L. bulgaricus* and *L. acidophilus* than would be expected to exist between members of one and the same species.

6. In addition to being unlike in their fermentative action upon the three sugars mentioned, they differ in another very important respect, namely in that *L. acidophilus* can and does live and develop in the intestine of man and animals, whereas *L. bulgaricus* is unable to do so.

7. The differences noted between these two types constitute evidence enough for classifying them as separate species. However, because of the various close relationships, it is suggested that these two types be placed in one and the same species of which *L. acidophilus* is the central type and *L. bulgaricus* a variant.

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PLATE 1

FIG. 1. L. bulgaricus from a twenty-four hour broth culture (strain F).
FIG. 2. L. acidophilus from a twenty-four hour broth culture (strain R-1-1).
FIG. 3. L. bulgaricus from a twenty-four hour milk culture (strain F).
FIG. 4. L. acidophilus from a twenty-four hour milk culture (strain R-1-1).
FIG. 5. "X" type agar colonies of L. acidophilus (strain R-1-5).
FIG. 6. "X" type agar colonies of L. acidophilus (strain D).
FIG. 7. "Y" type and disc-like agar colonies of L. acidophilus (strain R-1-1).
FIG. 8. Agar colony of L. bulgaricus (strain B-12).
FIG. 9. Agar colony of L. acidophilus (strain S-d).

Fig I

Fig II

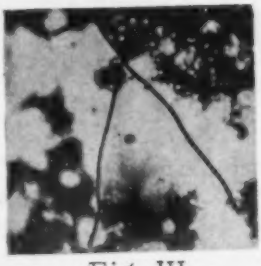

Fig III

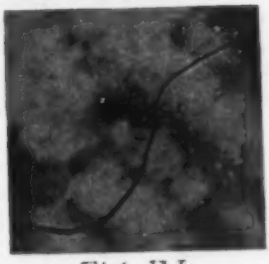

Fig IV

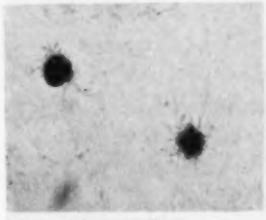

Fig V

Fig VI

Fig VII

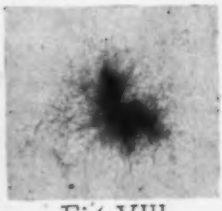

Fig VIII

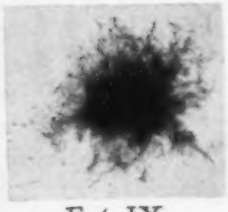

Fig IX

(Kulp and Rettger: Lactobacillus acidophilus and bulgaricus)

THE VARIATION IN SIZE OF LYTIC AREAS AND ITS SIGNIFICANCE

PHILIP HADLEY

Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

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One of the outstanding characteristics of the phenomenon of transmissible bacterial autolysis is the production of areas of lysis on a background of otherwise homogeneous culture. This occurs when the lytic agent, in appropriate dilution, is allowed to act in a bacterial film with which it is simultaneously seeded on slanted agar or on agar plates. These lytic areas, or plaques, are usually circular and may vary in size from pits of microscopic dimensions to eroded fields possessing a diameter of 18 to 20 mm. In case of the bacteriophage of d'Herelle acting on the Shiga dysentery bacillus the maximum size is 5 to 6 mm. Other lytic filtrates acting upon their appropriate cultures may show lytic erosions either larger or smaller.

Regardless of whether the lytic action arises "in the bacterial cells themselves," it is evident that the *exciting cause* of transmissible autolysis may exist outside of the bacterial cell and may be present in sterile filtrates of bacterial cultures, lysed or unlysed. If the bacterial culture on which the exciting substance acts remains physiologically stable and the medium upon which growth occurs remains unchanged, any variation in the manifestation of lytic action, as expressed by the characteristic lytic areas in that culture mass, might justifiably be attributed to variation in the exciting substance. It might perhaps assist in ascertaining what the exciting substance is, or what it is not, if some knowledge could be gained regarding its expressions of variability.

That the exciting substance can vary quantitatively (or numerically, according to d'Herelle) is well recognized as a result of numerous experiments. That it may also vary quali-

tatively has been suggested by many experiments involving the so-called adaptation of a lytic agent to bacterial cultures which, at the beginning, are only slightly influenced by it, but which, in the end, succumb to its lytic influence. But it also appears that the same exciting substance may show variations at different times in its power of causing lysis in one and the same culture and on the same culture medium. It is the object of the present paper to report certain observations on the variation in the behavior on the lytic agent at different times on the same strain of the Shiga dysentery bacillus.

The lytic agent used was received by Dr. Novy from d'Herelle in March, 1922. Owing to an accident in transit it was necessary to isolate the bacteriophage from the sawdust in which the tube was packed. In tests upon the laboratory stock strain of the Shiga dysentery bacillus the agent was demonstrated to be fully active, producing, after a few feedings and transfers, both inhibition and lysis in dilutions of 1×10^{-13}. In enumeration tests carried out more or less in accordance with the d'Herelle method, it formed in appropriate dilutions characteristic lytic colonies or plaques, on the surface of agar slant cultures.

The exact method of securing the lytic areas was as follows: One-hundredth cubic centimeter of a 5×10^{-11} dilution of the lytic filtrate in infusion broth of reaction pH 7.6 was added to an eighteen- to twenty-eight-hour culture of the dysentery bacillus. From this 3 loops were transferred, at intervals up to four hours, to agar slants, and these were incubated over night. Although cultures transferred at the four-hour period usually gave complete lysis, through the confluence of many lytic areas, some of the slant agar tubes revealed discrete lytic areas. These were circular and of two sizes, "large" and "small," without intergrading or intermediate sizes. The large areas were about 5 mm. and the small areas about 1 mm. in diameter. The small areas were slightly the more numerous, and both large and small plaques were evenly distributed over the slant agar surface. The small areas remaining after incubation of eighteen hours never developed into the large areas.

In March, 1922, in connection with another purpose, a test was set up in order to ascertain the inhibition titer of the stock lytic filtrate. The tubes contained at the start 2 cc. of infusion broth, one loop of twelve-hour Shiga culture and amounts of sterile lytic filtrate varying from 0.4 to 0.0004 cc. At the end of eighteen hours all the tubes showed complete inhibition. They were then sealed in glass and laid away in a dark box kept at room temperature. As the time of sealing the same lytic filtrate was giving regularly the large lytic areas. These sealed tubes will be referred to subsequently.

Subsequent to March, 1922, the study of several aspects of the lytic phenomenon was continued with the same strain of bacteriophage used in the test mentioned above, the culture being transferred from tube to tube, and sometimes submitted to culture feedings. As time passed, however, it was observed that the enumeration tests showed a gradually decreasing size of the lytic areas; so that, at the end of a year, all the areas were of the minute type—about 1 mm. in diameter. Various means were then tried to cause a return to the large size. A new culture of the central stock of the dysentery bacillus was secured. New strains of the Shiga type were secured from other laboratories and tested. Enumeration tests were performed on other types (Kruse, Strong) of the dysentery bacillus. The filtrate was passed rapidly through a series of broth cultures. New culture media were prepared and tested. The reaction of the medium was varied on both the acid and the alkaline side. The thickness of the bacterial layer was modified by changing the density of the Shiga suspensions. The amount of agar in the medium was varied. The thickness of the agar was increased and decreased. The results were invariably the same: The lytic agent was active in producing inhibition and lysis but the lytic areas remained of the small type.

On March 19, 1923, one year after the tubes mentioned above had been sealed, one of them was opened, a dilution was made and one loop inoculated into a young broth culture of the same Shiga dysentery bacillus previously used. From this, after varying intervals up to four hours, 3 loops were streaked on slanted

infusion agar and incubated over night. When the tubes were examined in the morning the large lytic areas were present, many of them measuring 6 mm. in diameter. Other sealed tubes have been opened from time to time since March, 1923, and it now becomes apparent that after nearly two years the sealed lytic agent has not changed in its power to yield large areas, while the stock lytic agent, undergoing frequent transfers and feedings is characterized by marked "degeneration."

An attempt was made to study the "hereditary trend" of cultures of the lytic agent obtained from the "small" and the "large" lytic colonies. We may recall that, according to the view of d'Herelle, each lytic plaque represents the result of development from a single corpuscular lytic unit, so that lytic cultures established from the discrete lytic colonies would represent "pure lines" of the lytic agent. It seemed possible that these pure lines might harbor intact the size-character of the lytic area. Accordingly, broth tubes lightly seeded with young, normal Shiga culture, were inoculated from the needle point touched to the lysed surface of the small and the large lytic colonies, respectively. From these tubes, growth in which was inhibited from the first, lytic cultures were developed, each of which, theoretically at least, had its origin in a single corpuscular lytic unit. When, after a brief enhancement of the lytic principle in each set by the feeding method, the "small" lytic culture and the "large" lytic culture were submitted to enumeration tests, it appeared that the "small" strain of filtrate gave rise to small lytic areas only, while the "large" lytic culture gave rise to both small and large lytic colonies in about the same number—or possibly with a slight preponderance of the larger type. These results may have a bearing on another observation made by the writer[1] on the behavior of the lytic (lysogenic) and non-lytic (resistant) colonies of *Bacillus pyocyaneus* (bacterial colonies in this case) when submitted to further plating. The non-lytic colonies always give rise to non-lytic colonies, while the lytic colonies give rise to both lytic and non-lytic. What relation, if any, exists

[1] Transmissible lysis in Bacillus pyocyaneus, Jour. Inf. Dis., 1924, 34, pp. 260-304.

between these phenomena is not at present clear. It might be that the resistant colonies of *Bacillus pyocyaneus* "carry" the physiological analogue of the "small" lytic agent (it is established that, though non-lysing, they carry *some* lytic agent), while the lytic colonies of pyocyaneus carry the analogue of the "large" lytic agent.

Whatever may be the explanation of this change in the mode of action of the bacteriophage in effecting the formation of lytic areas, one can at least regard it as a modification or as a variation; and as such it possesses significance. One is naturally tempted to place upon such a phenomenon an interpretation involving "loss of virulence" and that sort of thing, as d'Herelle has done; or perhaps to attribute it to changes in the amount of food substance in agar layers or different thickness, or to the accumulation of soluble anti-lytic substances in the agar substratum. D'Herelle,[2] for instance, states that the lytic areas are larger when the suspension of the bacteria is weak and smaller when the suspension is heavy. Also that they are larger when the adjacent medium is thicker and smaller when the medium is thinner. He says that upon a Petri dish where the agar layer is essentially the same thickness throughout, all the plaques of a given culture are of approximately the same diameter.

While it can scarcely be doubted that the fortuitous position of a lytic area upon slant agar, as well as many other things, may modify its size, the explanation presented by d'Herelle cannot hold in the present case. Neither the position of the colony nor the heaviness of the suspension, nor the thickness of the agar substratum, nor the constitution of the medium plays a part, as can be shown by a variety of tests. No manipulation of the medium nor of the method served to produce the large out of the small type of lytic colony. Regardless of whether we regard the variations in size as due to changes in "virulence," with the inherent implication of a virus-like nature of the lytic agent, it is clear that the exciting cause of lytic action is something susceptible of variation in one of its outstanding characteristics, while other

[2] d'Herelle, The Bacteriophage, p. 46.

factors in the reaction remain apparently stable. If, however, the diminishing size of the lytic areas is to be retarded as a loss of "virulence" in the d'Herelle sense, the most curious feature attaching to such an interpretation is that the old, sealed culture of the bacteriophage maintains its virulence while the virulence of the passage strain may become degraded through the influence of laboratory manipulation, accompanied by intermittent cultivations for a year in the cells of its proper host, the Shiga dysentery bacillus.

In this connection it may also be noted that there exists something of an analogy in the case of cultures of *B. pyocyaneus* of the lytic type. Old, sealed cultures containing the lytic agent permit the recovery of the lytic type of culture unmodified after more than a year, while the passage strain of pyocyaneus (which is characterized by simultaneous culture growth and lysis in the same tube) shows, within this time and after many generations on agar slants, a tendency to lose the lysogenic, and with it the pyocyanigenic, power. Thus, in this case also, one observes a change in the mode of expression of the lytic agent which may concern a variation in the exciting substance as well as an increased resistance of the culture to lysis.

Taking these observations into consideration, combined with certain of the experimental data presented by d'Herelle, one cannot close his eyes to the view that, whatever may be said on other grounds against d'Herelle's conception of the bacteriophage as a living, filterable virus, the inherent variation of energy in action which occurs in the exciting substance appears to be of a nature similar to variations especially correlated with complex organic matter, and most often with living protoplasm.

Finally it should be added that sealed tubes of the lytic agent opened in February, 1924, after being sealed for nearly two years, showed no reduction in the size of the lytic areas produced, while the tendency of the passage strain of the bacteriophage, originating from the sealed tubes opened in March, 1923, to manifest diminished size in its lytic areas has again become apparent.

SUMMARY

1. A strain of the d'Herelle bacteriophage active against the Shiga dysentery bacillus originally showed two types of lytic colonies—large and small, with no intergradations.

2. The passage strain of the lytic principle, conducted in series through many cultures during the course of a year showed at the end of this time only the small type of lytic colony.

3. On the other hand, sealed tubes of the lytic agent, opened at the end of one year, and again after nearly two years, showed the large lytic colonies quite intact.

4. Enumeration tests performed with lytic cultures of the "small" and the "large" lytic agents, respectively, showed that (*a*) the "small" lytic culture produced small areas only, while (*b*) the "large" lytic culture produced both small and large areas.

5. These results are regarded as suggestive that the exciting cause of transmissible bacterial autolysis is susceptible of independent variation quite apart from those variatons in its behavior which are admittedly determined by differences in the resistance of the bacterial culture on which it acts.

a'

A METHOD OF STAINING LYTIC AREAS PRODUCED BY THE BACTERIOPHAGE

PHILIP HADLEY

Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

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In carrying out the so-called "enumeration test" of d'Herelle with the bacteriophage working in its appropriate culture film on an agar substratum, it frequently happens that the lytic colonies of the "small" type[1] are difficult to observe, especially if one or more secondary or resistant colonies arise at the site of lysis and partially fill in the area, as sometimes happens. This is more likely to occur if the lytic agent is weak in energy or if the culture mass is to some degree resistant. The staining method to be described has the advantage (1) of staining the areas of lysis so that they can be easily seen, and (2) of revealing colonies of secondary or resistant culture arising on the lysed areas.

In brief, the method involves pipetting about one cc. of a well polychromed, Loeffler's methylene blue over the agar slant culture and tilting the tube so that the stain comes in contact with the bacterial film for about one minute. The stain is then washed out by adding distilled water with a pipette, blowing the stream with some energy over the slanted surface. The tube may then be rinsed with more water, or immersed in a jar of mercuric chloride (1:1000) and dried with a cloth. This procedure serves to stain the lysed areas red or reddish violet while the unlysed areas are either colorless (the color of the agar substratum) or a faint greenish blue. When stained in this manner the areas of lysis, viewed by transmitted light, stand out much more clearly than in unstained preparations. Moreover, some

[1] Hadley: This Journal, p. 397.

lytic areas that may easily be overlooked in unstained tubes are now detected. These effects are seen to best advantage in the case of the d'Herelle bacteriophage acting upon the Shiga dysentery bacillus.

Although d'Herelle[3] has stated that the areas of lysis are always sterile, and that at no time do there exist within them microscopically visible cells, in the experience of the present writer, a secondary culture of the organism frequently arises in the form of minute colonies on the lysed surface. These usually appear only after some days. Although the primary, unlysed culture does not stain on the agar slant, the secondary colonies, at the end of the staining process, appear as bluish or bluish purple points against the red lytic areas. These, in turn, contrast with the greenish blue background of the agar. The nature of these blue colonies will be referred to again subsequently.

One may gain some knowledge of the manner in which this staining process works by staining in a similar manner (1) a slant agar tube carrying a growth of normal Shiga culture, and (2) a sterile tube of slant agar of the same composition. In the first case, after washing the slant with distilled water or mercuric chloride, the entire surface of the agar formerly covered with culture shows the greenish blue color. In the second case the agar surface stains a bluish purple, not greatly different from the color of stained lytic areas. It is thus clear that the mere fact of staining of the lytic areas is not due to any modification of the agar surface resulting from lysis, since normal agar surface stains in a somewhat similar manner. Moreover, it is clear that the reason why the agar surface underneath the unlysed culture appears greenish blue after staining is because it remains relatively free from stain; in fact, it has been more or less protected against the stain by the covering of normal culture. When the tube is rinsed in water or mercuric chloride the normal culture is washed off, so that the only staining which the underlying agar receives is due to the brief contact with the stain during the process of washing. The color is greenish

[3] D'Herelle: The Bacteriophage, Philadelphia, 1922, p. 46.

blue rather than bluish because of the yellowish tone of the agar. This may, however, vary with reaction of the medium and with the length of the staining time.

Although the color of the stained lytic areas may resemble that of a stained normal agar surface, when not previously covered with culture, a difference can be detected. While the normal agar surface stains bluish purple, the lytic areas stain reddish-purple to red. There is a difference due to lysis. This difference suggests a difference in reaction at the lytic sites, as if these areas were more alkaline. In addition, the deeper tone of the stain in the lytic areas is probably due to the presence of alkaline debris remaining from the lysed bacterial cells.

The staining features of the secondary colonies of the dysentery bacillus call for further mention. It is recalled that the normal, unlysed culture washes off the agar surface when the tube is rinsed in water or mercuric chloride. The secondary growth, however, is not so affected; it remains adherent and stains blue to bluish-purple. One can see the colonies as bluish points against the red areas of lysis, which in turn contrast with the greenish blue of the agar background. In explanation of this difference, all that can be said at present is that the resistant colonies are strongly adhesive to the agar substratum. They cannot be washed off and are removed only with difficulty by the aid of a platinum needle. Indeed they differ in many respects from the normal Shiga culture, but the actual cause of these changes is at present only a matter of conjecture.

In conclusion a few guard points may be mentioned: 1. Ordinary methylene blue and a non-polychromed Loeffler's methylene blue do not give the typical differential coloring; particularly they fail to differentiate the lytic areas. A polychrome methylene blue, originally divised by Novy and used for many years at the University of Michigan as one of the three solutions in staining by a modified Romanowsky method, has given results surpassing in clearness those obtained by the ordinary polychromed methylene blue.

2. For good differentiation the first wash water must be poured off quickly before the denuded agar surface can take up the diluted stain.

3. Staining is more intense if the stain is heated in a tube to about 60°C. before added to the agar.

4. The stain must not run down the sides of the tube between the agar and the wall. This may be prevented by heating the line of junction of agar and glass with a fine blast lamp flame, and then cooling.

5. The results are clearer when staining is carried out twelve to twenty four hours after the lytic areas have attained their maximum size.

SUMMARY

1. A method is described for staining areas of lysis produced by the bacteriophage acting upon a bacterial film on agar slants.

2. The method serves to turn negative pictures of lytic colonies into positive ones, and to reveal for enumeration some of the smaller colonies which might otherwise escape notice.

3. The method also reveals the presence of colonies of the secondary or resistant strain of the lysed culture arising on the lytic sites.